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13. ABSTRACT (Maximum 200 Words)

Currently, we lack information on the role of the men-only chromosome, the Y chromosome, on prostate cancer. This project is designed to address this problem and to identify candidate gene s on the Y chromosome involved in this male-specific cancer. The objectives are: 1) to study the expression of Y chromosome genes in prostate cancer and 2) to evaluate their effects in over-expression in the prostate of transgenic mice. We have completed a survey on the expression of 31 Y chromosome genes in prostate cancer and have identified TSPY gene to be the most likely one to play a role in male oncogenesis. We have isolated and sequenced several polymorphic TSPY transcripts in normal and cancerous prostate samples, demonstrated that over-expression of TSPY potentiates cell proliferation in vitro and tumor formation in vivo. TSPY interacts with the mitotic cyclin B. Its expression is influenced by androgen. These studies have provided important information on the role of the TSPY gene and the Y chromosome on prostate cancer development and treatments.

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INTRODUCTION

The role of the Y chromosome in prostate cancer has been debated on numerous occasions (see reference 1 for review). Both a gain and a loss of this chromosome had been reported in the literature, suggesting the possible existence of an oncogene and tumor suppressor gene respectively. In the past, studies on the function of the Y chromosome in oncogenesis of male-specific organs, such as the prostate gland and testis, are limited due to the lack of information on the genetic content of this chromosome. Recent advances in the Human Genome Project have identified most of genes postulated to be on this chromosome, thereby providing the necessary resource for detailed studies on the contribution of Y chromosome genes to male-specific cancers, particularly on prostate cancer (2).

There are two objectives for the present project. First, we evaluate the potential roles of selected Y chromosome genes in prostate cancer by studying their expression patterns on a panel of prostate cancer samples with various degrees of malignancy. Results from these studies, together with information on the properties of the proteins encoded by the Y chromosome genes, should be helpful in determining the most likely candidate genes on this chromosome that may contribute to or be influenced by prostatic oncogenesis. Second, the function(s) of the most significant Y chromosome gene(s) in prostate cancer will be evaluated using a regulated system for transgene expression using nude mice and transgenic strategies. For the past 4 years, we have conducted studies according to the above objectives, and those modified thereof as discussed in Year 1 Progress Report. These studies demonstrated that TSPY gene is the most likely oncogene on this male-specific chromosome. Further characterization of its biochemical and physiologic properties suggests that TSPY is a cell cycle regulation, interacts with a key cyclin, and exerts proliferative activities when ectopically expressed in cultured cells and whole animals. These results strongly support the hypothesis that TSPY, and hence the Y chromosome, plays a critical role in prostatic oncogenesis. The significant results and achievements of this project are summarized in the following sections.

BODY

Task 1. To determine the expression patterns and probable functions of the Y chromosome genes in prostate cancer.

Through a series of expression studies conducted on 31 genes on the human Y chromosome, at the initial phase of this project, we have identified the repeated gene, testis-specific protein Y or TSPY, to be a significant candidate that potentially has a role in prostatic oncogenesis (2) (Appendix 1). This gene is differentially expressed in various prostate cancer samples and is inducible with androgen in the prostatic cancer cell line, LNCaP. TSPY has been mapped to a region of the human Y chromosome postulated to harbor a cancer predisposition gene, termed gonadoblastoma locus on the Y chromosome or GBY. Gonadoblastoma develops with high incidence (>30%) in XY sex-reversed individuals or patients with Turner syndrome harboring some Y chromosome materials (3). These patients have either a non-functional or a deleted male-determining gene, SRY, and hence develop into females, despite the presence of part or the entire Y chromosome in their genome. Deletion mapping has identified a small region of the Y chromosome containing the TSPY gene and predisposing these patients to gonadoblastoma development. Expression studies had indeed demonstrated a TSPY expression in tumor tissues of gonadoblastoma (Figure 1). Further, TSPY was also detected at high levels in testicular cancer of germ cell origins (3,4) (Appendix 2). These results suggested that TSPY is a putative candidate for GBY. Additional studies, conducted under the present research project, demonstrated that TSPY is indeed expressed in prostate cancers of various degrees of malignancy

(Appendix 3). These studies further supported a role for TSPY in prostatic oncogenesis. Because of the successful completion of the proposed studies under Task 1, we have proposed to further investigate the TSPY gene in oncogenesis, as outlined in the Year 1 Progress Report.

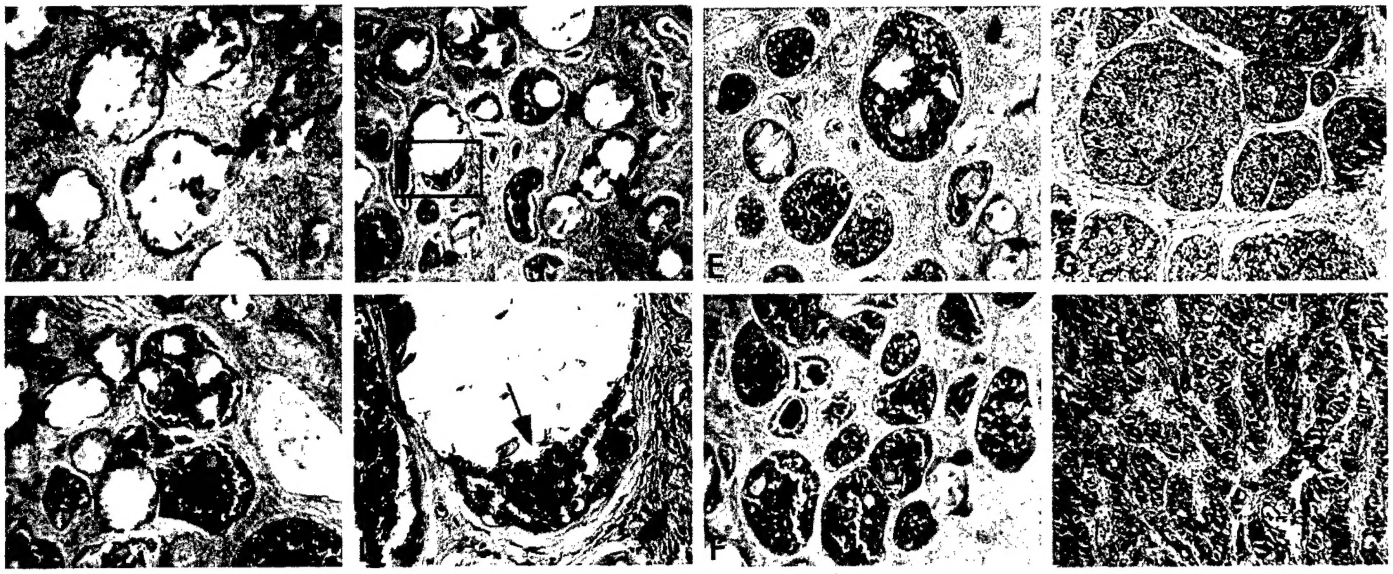

Figure 1. TSPY expression in various stages of gonadoblastoma detected by immunohistochemistry. XY individuals lacking a functional SRY gene develop into phenotypic females with dysgenetic or streaked gonads. Empty follicles are present in their gonads (A). At early ages, malignant germ cells, i.e. B and C) develop from the peripheral basal epithelia (arrow, D). Such tumorigenic growth expands to fill up the entire follicles (E and F). The follicle-confined growth persists in later stages (G) that eventually develop into more loosely arranged clusters of germ cell tumors. Presumably, these latter tumor germ cells might possess more aggressive properties leading to metastasis to secondary sites in the patients.

TSPY Expression in a Tissue Recombination Model of Prostate Cancer

In adult prostate, homeostasis is maintained via reciprocal stromal and epithelial interactions. During carcinogenesis, such reciprocal interactions are disrupted, favoring proliferation for the epithelial cells. Recently, excellent tissue recombination models of stromal-epithelial interactions have been established and used to study the hormonal and genetic determinants involved in normal and malignant prostatic growth. Once such model utilizes an SV40 T antigen-immortalized human prostatic epithelial cell line, BPH-1, and stromal cells from various sources (5). In general, such tissue recombinant with normal stromal cells, when transplanted to the kidney capsule of nude mice, produces solid branched epithelial cords and ductal structures resembling benign prostates. In testerosone and estradiol ($T+E_2$) treated hosts, such recombinants develop invasive carcinomas. Similarly, when human carcinomas-associated fibroblasts (CAFs)

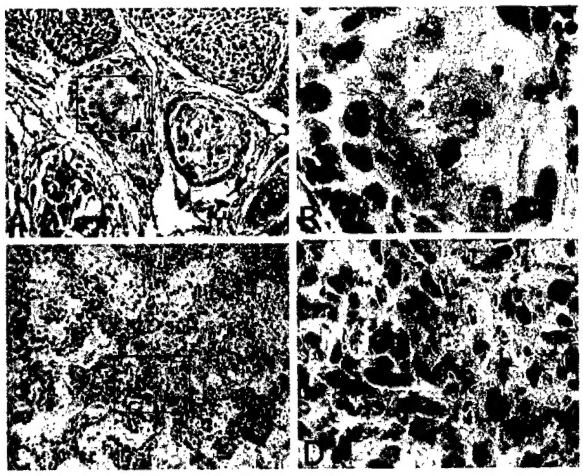

Figure 2. TSPY expression in A-B) BPH-1+rUGM & C-D) BPH-1+CAFs prostatic tissue recombnants.

from prostate cancer patients are used as stromal donors, significant prostatic cancer can develop in the tissue recombinants. These studies emphasize the importance of stromal microenvironment and hormonal influence in the carcinogenic transformation of the epithelial (BPH-1) cells. In collaboration with Dr. Gerald Cunha, UCSF and the developer of this tissue recombination strategy, we had examined the TSPY expression in recombinant transplants consisting of either BPH-1 and rat urogenital sinus mesenchyme (rUGM) or BPH-1 and CAFs. Our results demonstrated that TSPY proteins were expressed in the epithelial cells (i.e. BPH-1) in the pre-cancerous BPH-1-rUGM recombinants (Figure 2A, B). For the BPH-1 and CAFs recombinants, TSPY is widely expressed and is localized in the carcinoma and cancerous BPH-1 cells (Figure 2C, D). These observations suggest that TSPY is expressed in pre-cancerous BPH-1 cells and both cancerous epithelial BPH-1 and CAFs cells. If TSPY is involved in oncogenesis in these models, the former observation suggests that it might exert its effects in the early stages of this process while the latter observation suggests that such effects are needed to maintain an oncogenic phenotype for such tissue recombinants. These results are in agreement with those observed in $T+E_2$ induced prostate cancer in Nobel rats and further support the postulation that TSPY participates in male oncogenesis, including prostate cancer.

PROPERTIES OF THE TSPY GENE

The TSPY gene was independently isolated in my laboratory (6) and that of Jorge Schmidtke in Germany (7). Early studies demonstrated that the predominant transcripts were derived from transcriptional units of 2.8 kb in size consisting of 6 exons and 5 introns (6,8). It is tandemly repeated in 20.4-kb units for about 35 times in the short arm of the Y chromosome, representing approximately 0.7 MB of DNA (16). The TSPY tandems represent the most continuous repeating functional sequences, so far identified, in the human genome. TSPY has been postulated to serve a normal function in directing the spermatogonial cells to enter meiosis (3,8). Significantly, it shares tight protein homology to the SET oncogene, initially identified in a chromosome 9 rearrangement in a patient suffering from undifferentiated leukemia (9,10). The SET oncogene encodes a protein belonging to a family of cyclin B-binding proteins (11,12), including the nucleosome binding proteins (NAP1, NPL1). These proteins bind to mitotic cyclin B and contribute to the complex mechanisms of cell cycle regulation (10,12,13). Hence inappropriate expression of TSPY in either a female gonad (as in the case of gonadoblastoma) or the prostate gland (which normally expresses only a low level of TSPY) may be responsible for predisposition to and/or initiation of oncogenesis in these tissues. This hypothesis suggests that TSPY may function either as an oncogene or as an oncogenic promoter in both testicular and prostate cancers.

EVIDENCE SUPPORTING THE EXISTENCE OF POLYMORPHIC TSPY PROTEINS (Appendix 3)

Studies in both our laboratory and that of Jorge Schmidtke demonstrated three specific types of transcripts from the TSPY genes (6,8,14). The first one is a predominant transcript that encodes 309 amino acids. This transcript is designated as type 1 TSPY transcript. The cDNA initially isolated in our laboratory was derived from a transcript utilizing an alternate acceptor site, 11 nucleotides ahead of that at exon 6 for the type 1 mRNA, resulting in a protein with 295 amino acids. This minor transcript is designated as type 2 mRNA for TSPY. A variant type 1 transcript consists of an additional 18-nucleotide repeat within the first exon that is in-frame with the coding sequence, resulting in a protein with 315 amino acids. This rare transcript is designated as type 3 mRNA of the TSPY gene. All three types of transcript encode slightly polymorphic TSPY proteins that harbor a conserved (SET/NAP) domain homologous to that shared by the cyclin B binding proteins.

To determine if other forms of TSPY transcript may exist in normal and diseased tissues, we have recently examined the transcript population of TSPY in normal and cancerous samples of the testis and prostate gland. The entire coding sequences of different TSPY transcripts were amplified by PCR with specific primers from cDNAs synthesized from total RNAs of respective samples, cloned in plasmid vector and sequenced completely in both directions using an automated sequencer. The resulting sequences were then analyzed with the sequence analysis computer program, MacVector, and the BLAT program at the Genome Center, University of California, Santa Cruz. The BLAT program identifies the structural genes, including intron, exon and splice junctions, of the transcriptional units from the draft sequences of the entire human genome. Results from this study demonstrated a complex array of splice variants of TSPY transcripts in both prostatic and testis samples. They can be classified into two categories. The first one concerns the first exon in which a cryptic donor site at sequence immediately following amino acid residue #29 is used to splice into three different acceptor sites within exon 1 and exon 2. The first two variants splice into sequence preceding amino residue #118 and #135 respectively in exon 1 while the third one splices into that preceding amino residue #170 in exon 2. These in-frame cryptic RNA splices result in the deletion of 93, 110 and 145 amino acids from their respective ORFs. These transcripts are designated as variant Exon1A, Exon1B and Exon1C respectively. The splice variants on exon 1 are primarily type 1 transcripts. The second category of variant transcripts involves the skipping of the small introns, 3 and/or 4, in the RNA processing, resulting in altered reading frames beyond the additional sequences and slightly different proteins at the carboxyl ends. All encoded proteins, however, harbor the NAP domain in their respective ORFs. Specific primers flanking these new splice junctions had been used in RT-PCR analysis to confirm the presence of splice variants in both testis and prostate samples (data not shown). Although various forms of TSPY transcripts were present in both testis and prostatic samples, the shortened versions (e.g. Exon1A, Exon1B and Exon1C) of type 1 transcript were relatively higher in the prostates than the testis samples. This observation could be significant if the respective TSPY isoforms serve different/variable biological functions.

TSPY INTERACTS WITH THE MITOTIC CYCLIN B

We have adopted an in vitro binding and pull-down assay to study the possible interaction between TSPY and cyclin B. Initially, the human cyclin A, B1 and B2 were subcloned into a GST expression vector and expressed in bacterial hosts and purified from total cell extracts. The human TSPY cDNA was transcribed and translated in vitro using a TnT kit. TSPY is labeled with ^{35}S-methionine in the reaction mixture. To demonstrate an interaction, labeled TSPY proteins were reacted independently with GST-cyclin A, B1 or B2 fusion proteins. The GST fusion and bound proteins were purified by affinity binding to glutathione-conjugated resins and analyzed by SDS-PAGE. Results from this study demonstrated that TSPY is preferentially retained by GST-cyclin B1 or B2 fusion proteins, but not by GST alone nor GST-cyclin A (Figure 3A).

Previous studies demonstrated a spectrum of TSPY variant proteins in prostate cancer samples. To determine if these variant TSPY proteins do interact with the mitotic cyclin B similarly, we had subcloned the respective cDNAs of the TSPY proteins into an in vitro TnT vector. Radioactively labeled variant TSPY proteins were used in protein-binding assays with a GST- human cyclin B1 fusion protein. The results demonstrated that these variant proteins did bind to cyclin B1 and were co-precipitated with the cyclin B1 protein, but not GST alone (Figure 3B). Previous results suggested that alternative splicing events generate different transcripts deleting 92, 110 and 145 amino acids from the amino end of the predominant TSPY protein of 295 amino acids. These results suggested that the

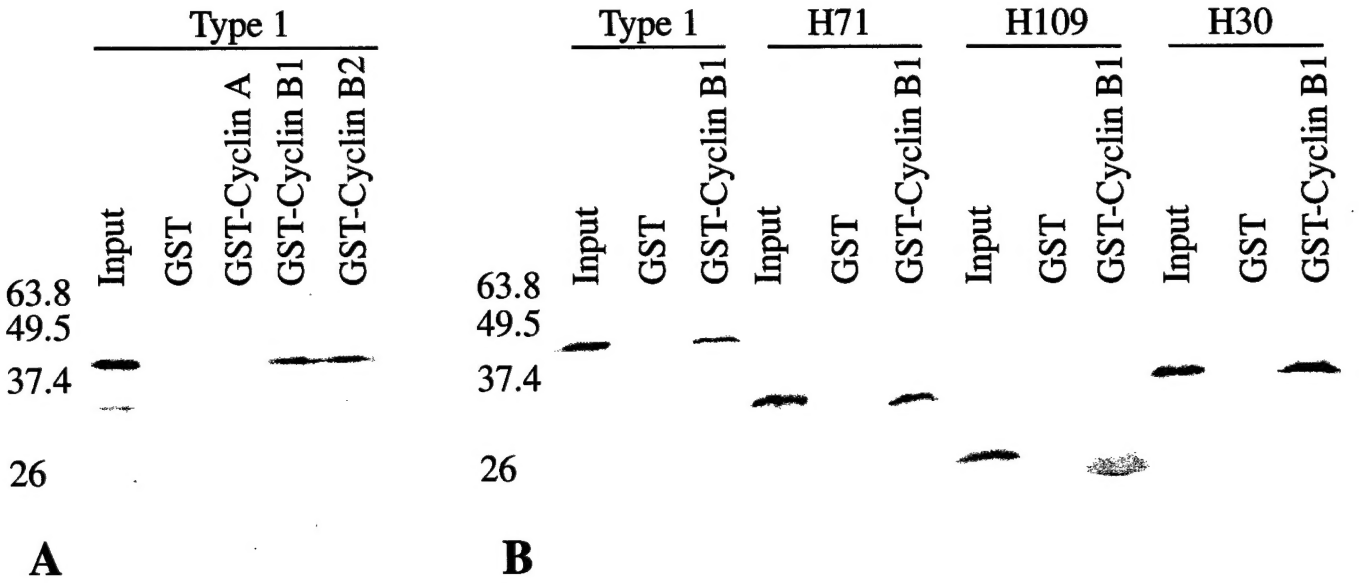

Figure 3. GST or GST-cyclin fusion proteins were induced in BL21(DE3) bacterial host by 1mM IPTG, then purified with affinity chromatography using glutathione sepharose. TSPY variants were synthesized by coupled transcription and translation reaction in the presence of ^{35}S-methinoine. GST-pulldown binding assay was performed with 40% TnT labelled products were used for each binding reaction at 4°C for 3 hours, washed 3 X with binding buffer. The bound proteins were then analyzed by SDS-PAGE autoradiography. **A.** Type 1 labelled-TSPY protein as a probe. The baits were cyclin proteins from *Xenopus*. **B.** Type 1, H71, H109, H30 were used as labeled TSPY probes. GST-human cyclin B1 fusion protein was used as a bait for the pull-down assays. Size markers are in kDa.

cyclin B binding domain is primarily located in the carboxyl end of the protein, as predicted by sequence alignment with NAP-1 and SET proteins. Although we are still uncertain on the functions of the different domains of the TSPY protein, deletions of portion(s) of its amino end might suggest a loss of certain function, assigned to this portion of the molecule while the cyclin B binding function is maintained in these shortened variants. It will be extremely interesting to define the functions of the different domains of TSPY and to evaluate what effects they may exert when they are deleted from the respective proteins.

Task 2. Functional evaluation of Y chromosome genes in prostate cancer

Initially we proposed to use a transgenic mouse strategy to test the oncogenic roles of Y chromosome genes identified to potentially play a role in prostatic oncogenesis. Our identification of TSPY gene as a significant candidate for the GBY gene on the Y chromosome and its likely role in prostate cancer have necessitated a refinement of our evaluation strategy, as outlined in the Year 1 Progress Report. We have adopted a strategy to address the probable mechanism of TSPY action in cell cycle modulation and its potential contribution to oncogenesis. Our working hypothesis is that TSPY is a specialized cell cycle regulator for male spermatogonial cells in the testis and when it is aberrantly expressed in male-specific organs, such as prostate and testis, it causes abnormal cell proliferation, leading to oncogenesis. To address this hypothesis, we used a cell culture system to determine the function of an over-expression of TSPY in cell proliferation using the tet-off system (15, as originally proposed). Once determined to have an influence in cell proliferation, an in vivo tumorigenicity assay using athymic nude mice was used to evaluate the consequence of such an over-expression of the TSPY gene.

OVER-EXPRESSION OF TSPY POTENTIATES CELL PROLIFERATION

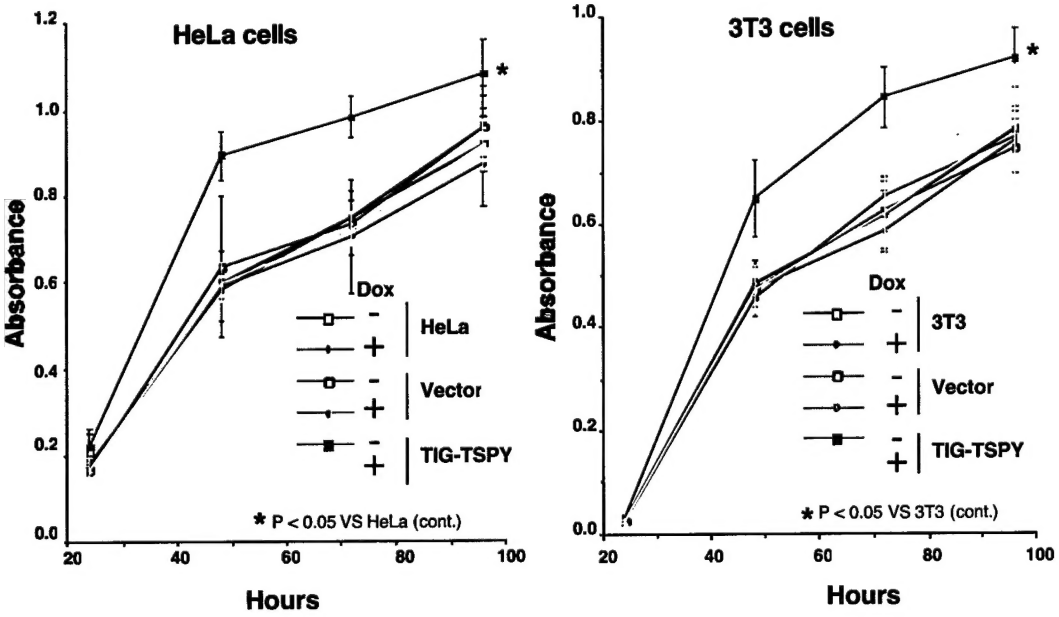

Figure 4. Over-expression of TSPY in HeLa (left) and NIH3T3 cells results in high cell proliferative activities compared to those with repressedTSPY gene.

The presence of the NAP domain in all TSPY variant proteins clearly suggests that this domain is very important for the biological function of this Y chromosome gene. Many of the cyclin B binding proteins are involved in cell cycle regulation. As demonstrated above, TSPY indeed interacts with cyclin Bs. In spermatogonial

cells, TSPY may exert its function in mediating these sperm stem cells in entering meiosis through such interactions. When it is aberrantly expressed in the prostate, it may potentiate a somatic cell proliferation, contributing to the multi-step oncogenic process. To examine this possibility, we have utilized the tet-off transgene regulation system (14) to manipulate the expression of a transfected TSPY (type 2) gene in cultured HeLa and NIH3T3 cells. In this system, both HeLa and NIH3T3 cells harbor a constitutively expressed transactivator gene that can transactivate a responder gene, consisting of responsive promoter and a bicistronic construct coding for both TSPY and a tracer green fluorescent protein (EGFP). In the presence of tetracycline or its derivative doxycycline, such transactivation is abolished, repressing the TSPY and EGFP expression. Hence, one can compare the effects of over-expression of the target genes between two identical cell populations cultured in the absence or presence of the antibiotic.

Several independent cell clones were isolated from stable transfection of HeLa or NIH3T3 tet-off cells using either the bicistronic construct, TIG-TSPY, or the corresponding vector, TIG, alone. Cells transfected with a functional TIG-TSPY construct and cultured in selective media without doxycycline gave a consistently higher number of colonies than those selected with media containing doxycycline or transfected with vector alone. Expression analyses of both EGFP (direct observation) and TSPY (immunofluorescence) demonstrated that both proteins were co-expressed in the same cells (data not shown). Their expression could be tightly regulated by doxycycline. Cell proliferation was analyzed using the XTT kit (Roche Biochemicals) that measures the enzyme activity of the mitochondrial succinate tetrazolium reductase, considered to be proportional to the number of metabolically active cells. Results from these analyses demonstrated that the proliferative activities of both HeLa and NIH3T3 cells increase to about 30-40% of the respective parental cells or cells transfected with vector alone (Figure 4). Such increases were abolished by addition of doxycycline in the media, resulting in repression of the responder gene in the cells. Hence, over-expression of TSPY potentiates cell proliferation in vitro.

OVER-EXPRESSION OF TSPY PROMOTES TUMOR GROWTH IN NUDE MICE

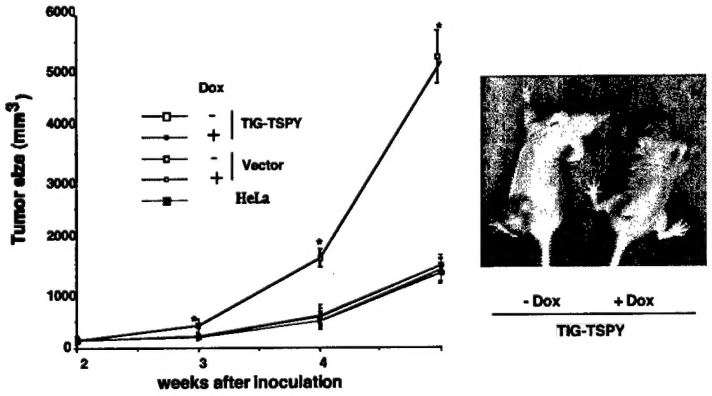

Figure 5. Over-expression of TSPY (-Doxy) promotes tumor growth in nude mice.

Figure 6. Direct visualization of tumor in nude mice.

The in vivo effects of TSPY expression were studied by tumorigenicity assays in nude mice. Both HeLa and NIH3T3 cell clones harboring either the bicistronic responder gene (TIG-TSPY) or the vector (TIG) alone were inoculated subcutaneously on the flanks of nude mice. Other animals were inoculated with the parental cells alone. They were fed either with or without doxycycline in their

drinking water. Six animals were used for each criteria point. Tumor sizes were measured weekly for 7 weeks. Results showed that tumors grew significantly faster in nude mice inoculated with HeLa tet-off cells harboring a over-expressed TSPY gene than in those harboring the vector alone (including EGFP). Nude mice harboring the same HeLa cells whose TSPY gene is repressed by feeding doxycycline-containing drinking water showed the same rate of tumor growth as those in hosts inoculated with HeLa parental cells or those with vector alone (Figure 5). Significantly, the NIH3T3 cells are not tumor cells. Under normal conditions, no tumor should result from such inoculation. It was indeed the case for mice inoculated with the NIH3T3 parental cells or those harboring the vector alone. But, for those inoculated with NIH3T3 cells over-expressing a TSPY gene, small tumors were observed in 5 of the 6 mice in the group fed with normal drinking water. Again for those fed with doxycycline-containing water, no tumor was observed even they were inoculated with the same cells. Expression analysis indeed confirmed the expression of TSPY and EGFP in animals fed with normal drinking water and repression of the transgene in those fed with doxycycline-containing water. Since GFP was expressed from the same transcripts with TSPY, the tumor growth could be monitored by direct observation of the nude mice under a blue light (Figure 6). This strategy should be globally application for other tumorigenicity assays. Results from this study suggest that over-expression of TSPY potentiates cell proliferation and promotes tumor growth in nude mice. Hence, TSPY is a putative proto-oncogene or oncogenic promoter gene on the human Y chromosome.

TSPY PROMOTER AND ANDROGEN REGULATION

To evaluate the regulation of TSPY gene in prostatic cells, a cell transfection and promoter analysis strategy was implemented. Various portions of the human TSPY gene promoter were subcloned in a luciferase expression vector, such that the TSPY promoter directly regulates the reporter gene (Figure 7A). The expression of the luciferase was then assayed in a cell transfection assay. A variety of host cells were used in these studies. They included prostatic cells, LNCaP (androgen responsive) and PC3 (androgen non-responsive), GC1spg (sprmatogonia-like) and GC2spd (spermatocyte-like), COS7, HeLa and NIH3T3 cells. Preliminary results from these studies suggested that only 160-bp upstream of the transcription start-site of the TSPY gene was sufficient in directing the expression of the luciferase gene in all cell types (e.g. Figure 7B). However, a bimodal expression pattern was observed in GC1spg, GC2spd and PC3 cells (Figure 7C, D, and E respectively). When additional upstream sequences (upto –2.4 kb) were included in the promoter, a reduction of the reporter activities was observed, suggesting that a repressor-like regulatory element(s) was present upstream of the core promoter (at –160 bp). Such expression pattern was absent in the HeLa female cells (Figure 3F). Since GC1spg and GC2spd and PC3 were derived from the testicular spermatogonial, spermatocyte and prostatic cells respectively, the bimodal regulation of the TSPY promoter in these cells suggested that they might resemble a regulatory mechanism in these male-specific cells, as compared to the more generic cells, such as HeLa cells.

TSPY expression was stimulated by androgen in the responsive cell line, LNCaP while its expression was not affected by the male hormone in the non-responsive cell lines, PC3 and COS7, even in the presence of a transfected androgen receptor gene. It will be interesting to determine the mechanism(s) by which TSPY is regulated by the male hormone that has been demonstrated to be important in the etiology of prostate cancer (10).

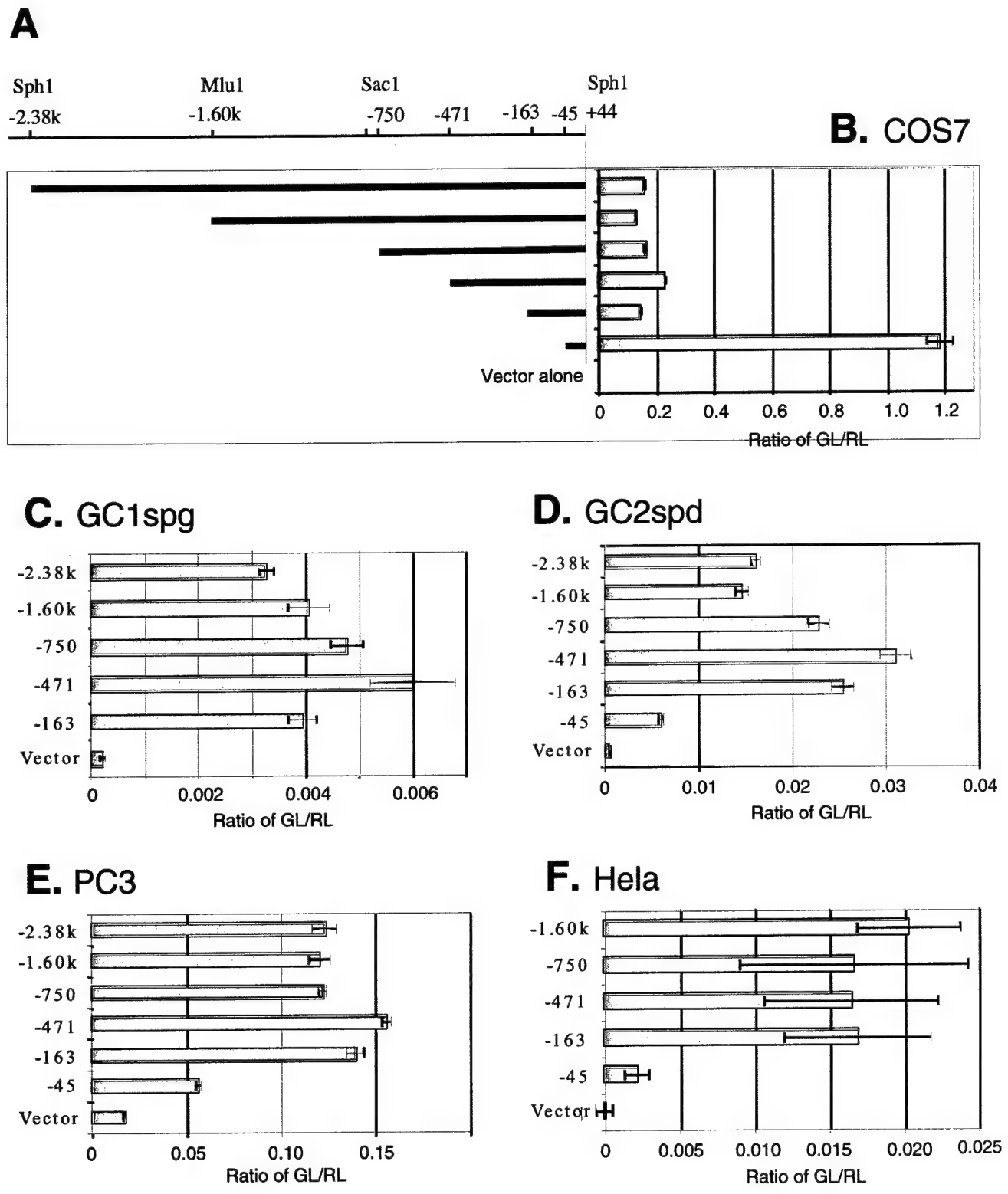

Figure 7. 0.3μg of pGL2-TSPY promoter or pGL2-basic was transfected into cultured cells with 15ng of pRL-TK (24 well plate). Transfection was performed by Fugene6 (Roche). After transfection, cells were incubated for 2 days. The promoter activity was measured by a dual luciferase assay kit (Promega). Values mean the ratio of tested promoter activity (GL)/internal control activity (RL).

TRANSGENIC MOUSE STUDIES OF TSPY GENE

Transgenic mouse studies are important components of the project. When targeted to prostate-specific expression, these studies can confirm the roles of the Y chromosome genes in prostatic physiology and diseases. For the past couple of years, we had implemented a tet-off transgene regulation system that allowed a manipulated expression or repression of the transgene in the host animals (11). This system constituted two components, a transactivator and a responder, by which the responder (target) gene could be regulated in bi-transgenic animals. We had successfully constructed various prostate-specific transactivator lines and responder lines. Currently, we are in the process of identifying the best combination of transactivator and responder lines for these experiments. Unfortunately, our progress had suffered somewhat with the unexpected departure of a key worker, Dr. Xing Xing Liu, in the early part of Year 3. Hence, this portion of the research was performed by the second postdoctoral fellow, Dr. Tatsuo Kido, who was also responsible for the protein and promoter work.

We had also generated transgenic mice harboring the native TSPY gene. Three transgenic founders were obtained. Two of the transgenic founders showed extreme abnormalities in their stature (much smaller than non-transgenic littermates) and died within two months after birth. The third founder seemed to be mosaic animal and never passed on his TSPY transgene to its offsprings. Although inconclusive in nature, these results suggested that abnormal TSPY expression might be detrimental to the health/development of the transgenic animals. Hence, a manipulated expression of the TSPY transgene, e.g. using the tet-off system, might be essential for these experiments. Using the tet-off system, we have generated numerous lines of transgenic mice harboring either the transactivator gene, PB-tTA, or responder, TIG-TSPY, transgene respectively. Numerous recombination of crosses between the transactivator and responder lines, as well as other reference lines, such as CMV-tTA and TIG, were performed, and the expressions of the respective responder transgene were analyzed by immuohistotochemistry and RT-PCR techniques. Our overall results were negative, i.e. we failed in finding the correct combination of prostate specific transactivator gene and responder gene that can be regulated by the presence and absence of doxycycline in the drinking water. These results seem to be frustrating, but not necessarily unexpected. Several groups of investigators, including that of Dr. Bruce Conklin at the Gladstone Laboratory, UCSF, have suggested that the success of the tet-off system of transgene regulation depends heavily on identifying the correct combination of transactivator and responder lines. Very often, this might require the tedious sorting out of numerous (>20) transgenic lines of each component for the identification of the right combination(s). In most cases, one of the two components, i.e. the transactivator line, has already been identified in other studies, e.g. by collaborators, so that one has only to identify the appropriate responder line for the studies. In our case, we have to identify both transactivator and responder lines. Further, despite intense efforts by other laboratories, no such tet-off transactivator line(s) has been reported, indicating the degree of difficulties in the task. Since then, we have adopted a different strategy of transgene activation using the Cre-Lox system. We now have preliminary results indicating that this system is very efficient in activating specific transgene in a tissue-specific manner. This study now constitutes a separate project for the evaluation of TSPY as a oncogene, particularly on prostate cancer.

Despite such set back, we believe that our alternate approaches using cell culture and nude mouse tumorigenicity assays clearly were more efficient in demonstrating TSPY as a significant proto-onocgene or tumor promoter gene on the Y chromosome. Its expression in prostate cancer correlates to the degrees of malignancy. The TSPY protein binds to cyclin B and when over-expressed

protentiates cell proliferation and tumor formation. Hence, our results from this project support the hypothesis that TSPY a putative oncogene and hence the Y chromosome containing TSPY gene might play a significant role in prostatic oncogenesis.

FUTURE DIRECTIONS

The TSPY gene on the human Y chromosome, as evidenced by the present studies, is a significant proto-oncogene or tumor promoting gene that plays critical roles in prostate cancer, as well a gonadoblastoma and testicular germ cell tumors. Further characterization of this gene, in terms of its normal functions, diseased mechanism, and biochemical and molecular properties will shed important lights on male-specific/Y-chromosome specific cancers. Further studies should provide significant insights in using this gene as diagnostic marker(s) and target for therapeutic strategies for these human diseases.

KEY RESEARCH ACCOMPLISHMENTS

- Conduct detailed studies on the expression of 31 Y chromosome genes in prostate cancer and cell lines.
- Identify the TSPY gene as a putative proto-oncogene residing on the Y chromosome.
- Provide evidence in support of TSPY as the gene for the gonadoblastoma locus on the Y chromosome.
- Detect the multiple TSPY variant transcripts encoding polymorphic proteins with potential different biochemical properties and functions.
- Demonstrate over-expression of TSPY leads to increase proliferative activities in cultured cells.
- Demonstrate that HeLa cells, an oncogenic cell line, over-expressing TSPY and inoculated in nude mice have a faster tumor growth than those whose TSPY gene is repressed.
- Demonstrate that the NIH 3T3 cells, a non-oncogenic cell line, induces tumor formation when inoculated in nude mice if their TSPY transgene is over-expressed while no tumor is induced in the hosts if their TSPY transgene is repressed.

REPORTABLE OUTCOMES

- First detailed study on the expression of all functional genes, isolated so far, on the human Y chromosome in prostate cancer. This report provides the clues for further studies on the contribution of the Y chromosome to prostate cancer.
  Lau Y-FC and Zhang J (2000). Expression analysis of thirty-one Y chromosome genes in human prostate cancer. Molecular Carcinogenesis 27:308-321.
- Identification of TSPY as a significant candidate gene for the gonadoblastoma gene on the Y chromosome. This study demonstrates the elevated levels of expression of TSPY in gonadoblastoma and testicular seminoma, suggesting a role for this gene on the oncogenesis of respective organs.
  Lau Y-FC, Chou PM, Iezzoni JC, Alonzo JA and Komuves LG (2001). Expression of a candidate gene for the gonadoblastoma locus in gonadoblastoma and testicular seminoma. Cytogenetics and Cell Genetics, 91:160-164 (2000).
- Demonstration of a preferential and elevated expression of TSPY on prostate cancer. This study establishes the potential universal role of TSPY in male-specific cancers, emphasizing on prostate cancer.

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CONCLUSION

The initial rationale for the present project is that the Y chromosome is a male-specific chromosome harboring genes important for the physiology of man. Since prostate gland is a male-specific organ, some Y chromosome genes should play a role in its normal physiology. Any mutations and/or abnormal expression of such Y chromosome gene might contribute to diseased state(s), i.e. cancer, of the prostate. Our research started with a comprehensive survey on the expression of 31 genes (representing the majority, i.e. 85%) on this chromosome in a panel of prostate cancer samples and cell lines. The overall conclusion from this study suggested that TSPY is a critical gene on the male-specific chromosome potentially involved in the etiology of prostate cancer. Our subsequent studies demonstrated that TSPY is aberrantly expressed in prostate cancer and other male-specific, i.e. testicular seminoma or Y chromosome related, i.e. gonadoblastoma, cancers. The postulation of TSPY as an oncogene is further substantiated by its proliferative activities in vitro and tumor promoting in nude mice. Additional studies demonstrated that it produces a spectrum of alternatively spliced transcripts encoding for a variety of polymorphic proteins with different biochemical properties. Hence, these studies support the role of TSPY in prostate cancer and raise further interesting questions regarding its mechanism of actions in prostatic oncogenesis, thereby supporting the merit for additional investigation on this putative oncogene on the man-only chromosome in the human genome.

SO WHAT

Uncontrolled cell proliferation is a key characteristic of tumor growth. All results, as discussed above, indicate that TSPY play an intimate role in this process. The Y chromosome, as the man-only chromosome, hence might be involved in the etiology of prostate cancer. Understanding the contribution of this chromosome in oncogenesis should be significant in establishing the diseased process(es) and therapeutic strategies for this unique cancer in men.

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Expression Analysis of Thirty One Y Chromosome Genes in Human Prostate Cancer

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Rapid advances in positional cloning studies have identified most of the genes on the human Y chromosome, thereby providing resources for studying the expression of its genes in prostate cancer. Using a semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) procedure, we had examined the expression of the Y chromosome genes in a panel of prostate samples diagnosed with benign prostatic hyperplasia (BPH), low and/or high grade carcinoma, and the prostatic cell line, LNCaP, stimulated by androgen treatment. Results from this expression analysis of 31 of the 33 genes, isolated so far from the Y chromosome, revealed three types of expression patterns: i) specific expression in other tissues (e.g., *AMELY, BPY1, BPY2, CDY,* and *RBM*); ii) ubiquitous expression among prostate and control testis samples, similar to those of house-keeping genes (e.g., *ANT3, XE7,ASMTL, IL3RA, SYBL1, TRAMP, MIC2, DBY, RPS4Y,* and *SMCY*); iii) differential expression in prostate and testis samples. The last group includes X-Y homologous (e.g., *ZFY, PRKY, DFFRY, TB4Y, EIF1AY,* and *UTY*) and Y-specific genes (e.g., *SRY, TSPY, PRY,* and *XKRY*). Androgen stimulation of the LNCaP cells resulted in up-regulation of *PGPL, CSFR2A, IL3RA, TSPY,* and *IL9R* and down regulation of *SRY, ZFY,* and *DFFRY*. The heterogeneous and differential expression patterns of the Y chromosome genes raise the possibility that some of these genes are either involved in or are affected by the oncogenic processes of the prostate. The up- and down-regulation of several Y chromosome genes by androgen suggest that they may play a role(s) in the hormonally stimulated proliferation of the responsive LNCaP cells. *Mol. Carcinog. 27:308–321, 2000.* © 2000 Wiley-Liss, Inc.

Key words: Y chromosome; gene expression; prostate cancer

INTRODUCTION

The human Y chromosome harbors a small number of genes that play essential and critical roles in the determination, differentiation, and maintenance of male specific organs, such as the testis and prostate gland. Recent positional cloning studies have isolated most of the small number of genes postulated to reside on the human Y chromosome [1–4]. These advances have provided invaluable resources for defining the functions of this chromosome in both normal and diseased physiology of the male-specific organs. Currently, 33 genes have been isolated from this chromosome and they can be divided into three groups (Table 1). The first group consists of X-Y identical genes located on the two pseudoautosomal regions (PARs). PAR1 is located on the telomere of the short arm while PAR2 is situated on the telomere of the long arm. PAR1 and PAR2 contain approximately 2.6 Mb and 400 kb of DNA, respectively. PAR1 harbors 10 genes and PAR2 has two genes. Each PAR gene has an identical gene on the corresponding PARs of the X chromosome. The second group consists of 10 X-Y homologous, but not identical, genes located on the nonrecombining region of the human Y chromosome (NRY). They are single-copy genes and most of which are ubiquitously expressed in many human tissues, including the prostate. Except the amelogenin Y (*AMELY*) gene that codes for an enamel protein, the functions of these genes are still largely unknown. Further, it is still uncertain whether these X and Y homologues are interchangeable in their functionality. The third group has 11 members that are specific to the human Y chromosome. The sex determining region Y (*SRY*) gene has been demonstrated to be the testis determining factor (TDF) that switches on testis differentiation pathway during embryogenesis [5]. Functions of the remaining 10 Y-specific genes have not been defined. Most of them consist of multiple copies on the Y chromosome and express in the testis. They are postulated to serve some functions in male-specific organs, such as regulation of spermatogenesis in the testis [6].

The role of the human Y chromosome in prostate cancer has not been investigated in details. Most studies utilized either classical or molecular cytogenetic techniques but molecular studies on individual genes on this chromosome have been lacking. So far, only the expression patterns of two genes,

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Table 1. Genes on the Human Y Chromosome*

Gene symbol	Gene name	Comments
Group 1: X-Y identical genes		
Pseudoautosomal region 1—short arm		
PGPL	pseudoautosomal GTP-binding protein-like	A conserved GTP-binding protein gene, closest to the telomere of the short arm of sex chromosomes
SHOX/PHOG	short stature homeo-box/pseudoautosomal homeo-box-containing osteogenic gene	A homeo-box containing gene involved in bone growth and stature. Postulated to contribute to Turner phenotype
XE7	XE7	A gene identified from an inactive-X cDNA library, nuclear located product
CSFR2A	GM-CSF receptor 2, alpha subunit	A receptor gene for the granulocyte-macrophage colony stimulating factor, a growth and differentiation factor
IL3RA	Interleukin 3 receptor alpha subunit	A cytokine receptor gene sharing homology to *CSFR2A* gene, IL3 binds to its receptors and promotes hematopoietic cell proliferation
ANT3	adenine nucleotide translocase	A member of the ADP/ATP translocase family, involved in cellular energy metabolism
ASMTL	ASMT-like	A gene encoding a putative fusion protein that shares homology with the bacerial *maf/orfE* at its amino end and to ASMT at its carboxyl terminus
ASMT	acetylserotonin methyltransferase	Coding for an enzyme involved in the last step of melatonin synthesis
TRAMP	TRAMP	A single-exon gene encodes a protein with homology to transposases of the Ac family
MIC2	MIC2	coding for a surface antigen detected by a monoclonal antibody, 12E7. (M, monoclonal; IC, Imperial Cancer Research Fund; 2, order of discovery)
Pseudoautosomal region 2—long arm		
IL9R	Interleukin 9 receptor	Receptor for a growth factor for T cells, erythroid and myeloid precursor cells
SYBL1	synaptobrevin-like 1	Coding for a synaptobrevin-like protein
Group 2: X-Y homologous genes		
Non-recombining Y region—short arm		
RPS4Y	ribosomal protein S4 Y isoform	Ribosomal protein gene postulated to be involved in Turner syndrome; X homologue, *RPS4X*
ZFY	zinc finger Y	Coding for a nuclear transcription factor harboring a DNA-binding domain with 13 zinc fingers, X-homologue, *ZFX*
PRKY	protein kinase Y	PRKY and its X homologue, *PRKX*, are members of cAMP-dependent serine/threonine protein kinase superfamily
AMELY	amelogenin Y	Expressed only in developing tooth buds. X-homologue, *AMELX*. Possibly related to tooth size locus
Non-recombining region—long arm		
DFFRY	Drosophila fat facets related Y	Homologous to *Drosophila* deubiquinating enzyme required for eye development and oogenesis. X homologue, *DFFRX*
DBY	dead box Y	Coding for a protein homologous to RNA helicases harboring conserved DEAD (Asp-Glu-Ala-Asp) motifs
UTY	ubiquitous TPR motif Y	Coding for a protein containing TPR motifs, implicated in protein–protein interaction and as a H-Y antigen
TB4Y	thymosin β4 Y isoform	X homologue sequesters actin
SMCY	selected mouse cDNA Y	Human homologue of the mouse *Smcy* gene coding for a minor histocompatibility H-Y antigen and may serve as a spermatogenic factor
EIF1AY	Translation initiation factor 1A	X homologue is an essential translation initiation factor

Table 1. (*Continued*)

Gene symbol	Gene name	Comments
Group 3: Y-specific genes		
Non-recombining region—short arm		
SRY	sex determining region Y	Evolutionary conserved gene coding for the testis determining factor (TDF)
TSPY	testis-specific protein Y-encoded	Repeated gene coding for a putative cyclin-B binding protein homologous to that of oncogene, *SET*. Postulated to direct the spermatogonial cells to enter meiosis and to be involved in oncogenesisi of the testis tumors and gonadoblastoma.
PRY	PTP-BL related Y	Coding for a protein homologous to PTP-BL, a putative protein tyrosine phosphatase, also repeated on the long arm
TTY1	testis transcript Y1	Repetitive transcripts without any protein coding sequences, also present on the long arm
TTY2	testis transcript Y2	Repetitive transcripts without any protein coding sequences, also present on the long arm
Non-recombining region—long arm		
BPY1	basic protein Y1	Coding for a 125-residue basic protein, repeated gene
BPY2	basic protein Y2	Coding for 106-residue basic protein, repeated gene
CDY	chromodomain Y	Coding for a protein with chromodomain and putative catalytic domain, may modify DNA/protein during spermatogenesis, repeated gene
XKRY	XK related Y	Coding for a protein homologous to XK, a membrane transporter protein, repeated gene
RBM	RNA binding motif	Repeated gene coding for a RNA binding protein, a candidate for the azoospermia factor (AZF)
DAZ	deleted in azoospermia	A gene coding for another RNA binding protein, a candidate for the azzospermia factor (AZF)

*See references 1–4,30 for detailed description of genes on the human Y chromosome and information on additional references.

CSF2RA coding for the α subunit of the GM-CSF receptor and *MIC2* coding for a cell surface antigen, on PAR1 [7], and two Y-specific genes, *SRY* and *ZFY*, had been studied in both prostate adenocarcinoma and benign prostatic hypertrophy (BPH) [8,9]. To initiate a survey of expression patterns of these Y chromosome genes, we have developed an experimental strategy to study the expression of a large number of genes in representative prostate samples and prostatic cell lines. Results from this study identify several Y chromosome genes that are either aberrantly expressed in prostate samples and/or regulated by androgen treatment in the responsive prostatic cell line, LNCaP. This survey hence has identified several candidate genes on the Y chromosome that may either play a role(s) in or be influenced by prostatic oncogenesis.

MATERIALS AND METHODS

Tissues, Cell Lines and RNA Preparation

Tissue biopsies were obtained from the Cooperative Human Tissue Network, Western Division at Cleveland. The prostate samples include one benign prostatic hyperplasia (BPH), two BPHs with cancer foci, and three prostate cancer specimens with various degrees of malignancy (Table 2). Their classifications were based on pathological examination of parallel preparations from the respective samples by attending pathologists of the Institute of Pathology at Cleveland. Since the prostate gland contains various amounts of epithelia and stroma, it is conceivable that normal epithelial cells and stromal elements might be present within the cancerous epithelia and vice versa in these samples. Two prostatic cell lines, LNCaP and PC3, were included in the analysis and they were obtained from the American Type Culture Collection. These cell lines cause tumor formation when they are injected into nude mice. The LNCaP cell line is androgen responsive and can be stimulated by male hormone treatment while PC3 is androgen non-responsive under the same conditions [10,11]. Three testis samples (two normal and one atrophic) were also included as references. Total RNAs were purified from respective tissues and cell lines according to a standard procedure by using the Trizol Reagent (GIBCO-BRL). Purified RNAs were treated with RNase-free RQ1-DNase (Promega Inc.) according to the manufacturer's protocol, phenol-chloroform extracted, precipitated with ethanol, and stored in DEPC-treated water at −20°C.

Table 2. Human Tissue Samples

Samples	Diagnosis
Testes	
1. Normal testis	25 year old from autopsy
2. Normal testis	34 year old with atrophic testis and chronic epidymitis
3. Atrophic testis	40 year old with seminoma on left testicle, uninvolved right testicle with atrophy
Prostates	
4. BPH	72 year old, BPH, no patient information
5. BPH + CA	69 year old, adenocarcinoma, Gleason's score 3 + 2 = 5, 25% CA, 50% NEO of remainder
6. BPH + CA	55 year old, Gleason's score, 3 + 2 = 5, 25% CA + 100% BPH on remainder
7. CA	66 year old, % CA unavailable, Gleason's score 3 + 4 = 7
8. CA	61 year old, infiltrating prostate cancer, Gleason's score 3 + 5 = 8, 100% CA in Tumor, 20% NEO of remainder
9. CA	69 year old, prostate cancer, 10% CA of tumor 90% NEO of remainder, other information unknown
Cell lines	
10. LNCaP (androgen responsive)	
11. PC3 (androgen nonresponsive)	
Control	
12. Blank	

CA, cancer; NEO, neoplasm; BPH, benign prostatic hyperplasia.

LNCaP and PC3 cells were cultured in RPMI1640 and Ham F-12 media, respectively, supplemented with 10% fetal bovine serum. They were grown at 37°C in a 5% CO_2 atmosphere. For androgen treatment, LNCaP cells were seeded at 2×10^6 cells per 150 mm culture dish and were cultured for 7 d in RPMI1640 medium supplemented with 10% charcoal-dextran treated (hormone-depleted) fetal bovine serum. The cells were then cultured separately for additional 9 d in the same medium supplemented with a synthetic androgen, R1881, at 0, 10^{-10}, 10^{-9}, or 10^{-8} M concentration. The culture media were changed every 2–3 d. The cells were then harvested for RNA preparation by using the Trizol Reagent (GIBCO-BRL) and RQ1-DNase digestion, as before.

RT–PCR Analysis

PCR primer sets were designed from cDNA sequences of 31 of the 33 genes from the Y chromosome retrieved from the GenBank database [1,2]. Table 3 shows the primer sets for 31 Y chromosome genes and five reference genes. The primers for the repetitive sequences, *TTY1* and *TTY2* were omitted. Their transcripts do not contain any protein-coding capability and were not studied in details here. In the cases of X-Y homologous genes, the respective primer sets were designed to be specific for only the Y-located alleles according to available sequence information. The primers were designed to amplify cDNA fragments of 400–600 bp in size and from the 3′ end of the respective transcripts of the Y chromosome genes. cDNAs were synthesized with 2 μg of total RNAs from the respective samples by using a Superscript II cDNA synthesis kit (GIBCO-BRL). Five percents of the cDNA products were amplified with the respective primer sets in 25 μL of reaction buffer by using the High Fidelity PCR kit (Roche Biochemicals). The touchdown PCR conditions were: 1 × 95°C, 5 min; 2 × (95°C, 30 s; 60°C, 30 s; 72°C, 90 s), 2 × (95°C, 30 s; 58°C, 30 s; 72°C, 90 s), 2 × (95°C, 30 s; 56°C, 30 s; 72°C, 90 s), 2 × (95°C, 30 s; 54°C, 30 s; 72°C, 90 s), 35 × (95°C, 30 s; 52°C, 30 s; 72°C, 90 s), and 72°C, 10 min. The reactions were performed in a multiplexing format on a 96-well microtiter dish by using the PTC-100 Programmable Thermal Control (MJ Research Inc.). Eleven samples and one blank control were arranged along the horizontal row for each Y chromosome gene. For a 96-well format, eight Y chromosome genes were analyzed. The touchdown procedure allowed PCR primer sets of different annealing temperatures to be used in the same time [12–13]. Fifteen microliter of the amplified products were then analyzed by electrophoresis in a 1.2% agarose gel, and visualized by ethidium bromide staining. For the relative expression levels of the Y chromosome genes in LNCaP cells under different hormonal treatments, the last round of amplification was adjusted to 20, 25, 30, or 35 cycles, depending on the relative abundance of the respective Y chromosome gene transcripts in the

Table 3. Primer Sets Used for RT–PCR Analysis

Gene	Primers	Sequences (5′ to 3′)
Y chromosome genes		
Pseudoautosomal regional—X-Y identical genes		
PGPL	PGPL-3	CGAGGTCCACCTTGTTGTGAAC
	PGPL-5	CATCATGGGGTCAGGAGAATCC
SHOX/PHOG	SHOX-3	CGACAAGAGCAAAACTCCATCTG
	SHOX-5	AGGACCACGTAGACAATGACAAGG
XE7	XE7-3	GGAGCATCAGACCATTTTGTACCAD
	XE7-5	CAGCGTACTTGGCACTTCAGTTTC
CSFR2A	CSFR2A-3	ATGTCCATGCCATTCCTACACC
	CSFR2A-5	TTCAACCCTCCCAGCAATGTC
IL3RA	IL3RA-3	ATTACACAGGCATCTCCCATGC
	IL3RA-5	AACCTCCTTCCAGCTACTCAATCC
ANT3	ANT3-F	CACCAAGTCCGACGGCATCCG
	ANT3-G	ACGGTTGAGGATTCTACGTGG
ASMTL	ASMTL-3	TGGGAGGTCAAACACAGTCACC
	ASMTL-5	GATATTGCCAGCAAAGTGGACG
ASMT	ASMT-3	CAATTACCAGAATGCCACCACC
	ASMT-5	AGGAAGGAACCAGTACCTGGAGAC
TRAMP	TRAMP-3	TGAAGAGAACGCTGGCAATGCTAC
	TRAMP-5	GCTTTCAGTCATTCAGGGAAACAC
MIC2	MIC2-3	CTCGGTGGGGTTGACATTTG
	MIC2-5	CACAGGAAAGAAGGGGAAGAGG
IL9R	IL9R-1	TGGAGCCCTTGTCTGAGACTGAAC
	IL9R-2	AGCTCTCAGTTTCCTGGAGCAAGG
SYBL1	SYBL1-3	GAGTTGACTGCTGACCGTATTCG
	SYBL1-5	TGTCGCCGCTAGTCTTATGAGC
Nonrecombining region—X-Y homologous genes		
RPS4Y	RPS4Y-1	AACCTCGGTCGTGTTGGTGTGATC
	RPS4Y-2	GCTGCTACTGCAATTTAGCCACTG
ZFY	ZFY-3	CATCAGCTGAAGCTTGTAGACACACT
	ZFY-5	ATTTGTTCTAAGTCGCCATATTCTCT
PRKY	PRKY-R	AAAACAGACAACATAAAATTACA
	PRKY-F	GACCTTTTCTTCACGTGAC
AMELY	AMELY-6	TGGCTGCACCACCAATCATC
	AMELY-7	GTGAGGCTGTGGCTGAAC
DFFRY	DFFRY-3	GGTGTAAACTGACAAAGATGGGCTC
	DFFRY-5	CCAGCAGCACATCACCTTGAACAAC
DBY	DBY-3	GCATTACTGAGCCAACAGGACATC
	DBY-5	AGGATTTGGTGCCAGAGACTATCG
UTY	UTY-3	GGTATGGTGAAGTTGGTGGTCTTG
	UTY-5	CAGATGCTGTTTCCAGTCCTAACC
TB4Y	TB4Y-3	GCTATTTTCCTTCCCTGCCAGC
	TB4Y-5	GGTGTCGCCTCTTTTTCTGTGG
SMCY	SMCY-3	AAGTACATAAAGAGGGTGGTGGGG
	SMCY-5	GCTTACAACACAAGGATTCAGGCTC
EIF1AY	EIF1AY-3	CGAGGCACAAAGGATGAAAAGG
	EIF1AY-5	GGTTCTACAGTTGGGATTTTGGC
Nonrecombining region—Y-specific genes		
SRY (Set A)	SRY mid-3	TCTTCGGCAGCATCTTCGCC
	SRY mid-5	TCGCGATCAGAGGCGCAAGA
(Set B)	SRY-coding3	GGTCTTTGTAGCCAATGTTA
	SRY-coding5	ATGCAATCATATGCTTCTGC
TSPY	TSPY4-3	CCTTGAGAATGTTTATTTTTCATTC
	TSPY4-5	CAGATGTCAGCCCTGATCACTG
PRY	PRY-3	TCCACTCAACGCCTCTCCTTTC
	PRY-5	GGTTATCTTCACAGTGCCTCGGAC
BPY1	BPY1-3	CCTAAACTTAGTTGCTGCTCAGGG
	BPY1-5	CGAAGAAGAAGACTACCAAGGTGG

Table 3. (*Continued*)

Gene	Primers	Sequences (5′ to 3′)
BPY2	BPY2-3	GCATTACCCAGTTTTTGCAGTCAG
	BPY2-5	CCAGATTTTCACAGGTGCTGCTTAC
CDY	CDY-3	GATCGTCAGTGGATTTTGAGCC
	CDY-5	GGTTCCAAACCCCTTATACGACC
XKRY	XKRY-3	GAAGCGGAGCTTAAAGCCAAAG
	XKRY-5	GCATTGCTGATGACATATTCCCTC
RBM	YRRM-13	TACTTTGGTCTTTTCTG
	YRRM-14	ATGATGGCTACGGTGAG
DAZ	DAZ-1	GGAGCAAAGGAGAAATCTGTGGAC
	DAZ-2	TCAAAACCAGCAACTTCCCATG
Reference genes		
Androgen receptor		
AR	AR-3	GTTTCCAATGCTTCACTGGGTG
	AR-5	ACCAATGTCAACTCCAGGATGC
Prostate specific antigen		
PSA	PSA-3	CAGTCCCTCTCCTTACTTCATCCC
	PSA-5	GCACCCCTATCAACTCCCTATTG
SET		
SET	SET-3	AGCTGAGTCCATTATCCCACCCAG
	SET-5	CGCACTTTTGCAGGATGACCTC
Uridine diphosphoglucuronosyltransferase		
UGT	UGT-3	TCTCCAAATGCTATCCTTCCCC
	UGT-5	TCTGGATTGAGTTTGTCATGCG
Hypoxanthine-guanine phosphoribosyl transferase		
HPRT	HPRT-A	CCTGCTGGATTACATTAAAGCACTG
	HPRT-B	GTCAAGGGCATATCCAACAACAAAC

LNCaP cells. The number of cycles was determined empirically for each gene. Such adjustments ensured a semilinear amplification of each Y chromosome gene. The resulting PCR products were analyzed similarly in 1.2% agarose gels. The ΦX174 RF DNA digested with HaeIII was used as molecular weight markers. The PCR primers of several Y chromosome genes, such as *TSPY*, *RBM*, and *DAZ*, spanned across one or several introns in their respective structural genes. In all cases, PCR amplification of the cDNA samples did not reveal any products specific for the respective genomic DNAs, suggesting that the RQ-DNase treatment was effective in eliminating any contaminant DNA in the original RNA preparations. Hence, all signals obtained from the touchdown RT–PCR analysis were derived from the respective Y chromosome gene transcripts.

Northern Blotting

Twenty microgram of total RNAs from respective LNCaP cells stimulated with the synthetic androgen, R1881, at different dosages were processed for northern hybridization, as described before [25]. cDNA fragments from RT–PCR amplification were subcloned into plasmid vector by using a TA Cloning kit (Invitrogen Inc.) and confirmed by single-passage sequencing with an ABI automated sequencer (DNA Sequencing Core Lab, Howard Hughes Medical Institute). The respective inserts were excised from the plasmids and labeled with a random prime labeling kit (Roche Biochemicals) in the presence of ^{32}P-α-dCTP (Amersham Inc.) and used as probes in the northern hybridization procedure. Hybridization signals were visualized by X-ray autoradiography.

RESULTS

To establish an expression–function correlation, we had developed a strategy for identifying potentially important Y chromosome gene(s) involved in prostate oncogenesis. In this approach, a semiquantitative reverse transcription and touchdown PCR assay [12,13] was used to analyze the expression of Y chromosome genes in a panel of representative prostate and testis samples. cDNAs were initially synthesized from total RNAs of prostate and testis samples and subjected to PCR amplification with respective gene-specific primers in a multiplex format. The touchdown procedure utilized an initial high annealing temperature that was gradually ramped down at decreasing intervals, thereby

allowing primer sets of different T_m to anneal to the correct sequences in the respective samples. For a 96-well microtiter dish, one could examine a total of 11 samples and one blank control (along the horizontal row) for eight gene primer sets (along vertical column). The RNA samples were derived from specimens with different clinical stages or cells under various culture conditions. This strategy, hence, allowed a rapid preliminary evaluation on the expression of a large number of specific genes on a panel of tissue/cell samples.

Expression of Y Chromosome Genes in Normal and Diseased Prostates

Of the 31Y chromosome genes examined, specific PCR products were obtained from the respective gene transcripts (Figure 1A–D). Genes residing on the pseudoautosomal regions were either expressed uniformly (e.g., *PGPL, ASMTL, IL3RA, ANT3, XE7, MIC2,* and *SYBL1*) or minimally (e.g., *SHOX/PHOG, CSFR2A,* and *IL9R*) (Figure 1A). These expression patterns confirmed their ubiquitous and tissue-specific expression, respectively. However, we did observe slightly higher levels of expression of *PGPL* and *CSFR2A* in the prostate cancer samples (#7–9) than in the BPH samples (#4–6). Further, we could not rule out the possibility that these tissue-specific growth factors and receptor genes, i.e., *PHOG, CSFR2A, IL3RA,* and *IL9R,* might be aberrantly expressed in other forms, e.g., metastatic, prostate cancer, or under androgen stimulation, (discussed below). The expression patterns of the X-Y homologous genes were more complex (Figure 1B). The *RPS4Y, DBY,* and *SMCY* genes were expressed relatively uniformly while the remaining six X-Y homologous genes were expressed variably among samples of the testis, prostate, and prostatic cell lines. There seemed to be relatively higher RT–PCR products for *ZFY, PRKY,* and *DFFRY* among the prostate cancer specimens (#7–9) than those among the BPH samples (#4–6) while the reverse was observed for *EIF1AY.* Presumably, these X-Y homologous genes are expressed in most human tissues [2]. Our results demonstrated that their levels of expression could vary between tissues and among samples of the same tissue. Their variability might reflect the physiological states of the respective tissues or samples.

The Y chromosome-specific genes are present only in the male genome and do not have any homologue and/or equivalent genes in the female genome. They are postulated to serve vital functions in male specific organs, such as male sex determination and regulation of spermatogenesis [5,6]. Initially all these genes were demonstrated to be expressed specifically in the testis [2]. Indeed, transcripts from *CDY* and *RBM* genes were primarily detected in the testis (Figure 1C). Both *RBM* and *CDY* had been postulated to serve some functions in spermatogenesis, their testis specific expression was reasonably expected. Apart from their expression in LNCaP cells, the *BPY1* and *BPY2* genes also exhibited a testis-specific pattern. They encode two small basic proteins of unknown functions. The expression levels of the remaining five genes in the testis and prostate samples were heterogeneous (Figure 1C), suggesting that they might represent aberrant expression patterns in these tumor tissues and cell lines. Several aberrant RT–PCR fragments were observed in PC3 cell line. Subsequent cloning and sequencing of these aberrant fragments, however, showed that they were not derived from transcripts of corresponding Y-specific genes. Similar analysis of the gene-specific fragments from other testis and prostate samples confirmed their origins from respective Y chromosome gene transcripts. The lack of Y-specific gene products in PC3 cells might reflect the fact that cytogenetic analysis had failed to reveal an intact Y chromosome in this cell line [11]. The detection of RT–PCR products from both pseudoautosomal and X-Y homologous genes in this cell line (Figure 1B) suggests that perhaps part of this chromosome might have been retained (or translocated to other chromosomes) in its genome. PCR amplification of genomic DNA isolated from PC3 cells indeed showed positive products from selected PAR, e.g., *ANT3, IL3RA,* and *SYBL1,* and X-Y homologous genes, e.g., *ZFY, DFFRY, DBY,* and *AMELY* (data not shown). However, we still cannot rule out the possibility that the RT–PCR products might be derived from transcripts of the respective X alleles or homologues.

Five reference genes, androgen receptor (*AR*) [14], prostate specific antigen (*PSA*) [15], oncogene *SET* [16], uridine diphosphogulucuronosyltransferase (*UGT*) [17] and hypoxanthine phosphoribotransferase (*HPRT*) were analyzed similarly with the touchdown RT–PCR technique by using respective gene-specific primers. The results indicated that both *PSA* and *SET* genes were expressed almost evenly in all prostate samples and the LNCaP cells (Figure 1D). The *AR* expression varied among the samples while that for UGT was predominantly in the LNCaP cell line. In particular, *UGT* gene was down-regulated upon androgen stimulation in this cell line [17]. Most testis and prostate RNA samples seemed to be of good quality, as indicated by their relatively uniform signals for *HPRT* (Figure 1E). The PC3 cells had been demonstrated to lack functional AR and PSA [10,11]. However, recent reports had documented the presence of the respective genes and residual expression of both androgen receptor and PSA molecules in PC3 cells [10,11,43,44]. The detection of both *AR* and *PSA* transcripts with the highly sensitive RT–PCR technique here confirmed the low-level expression of these genes in this androgen-insensitive prostatic cell line.

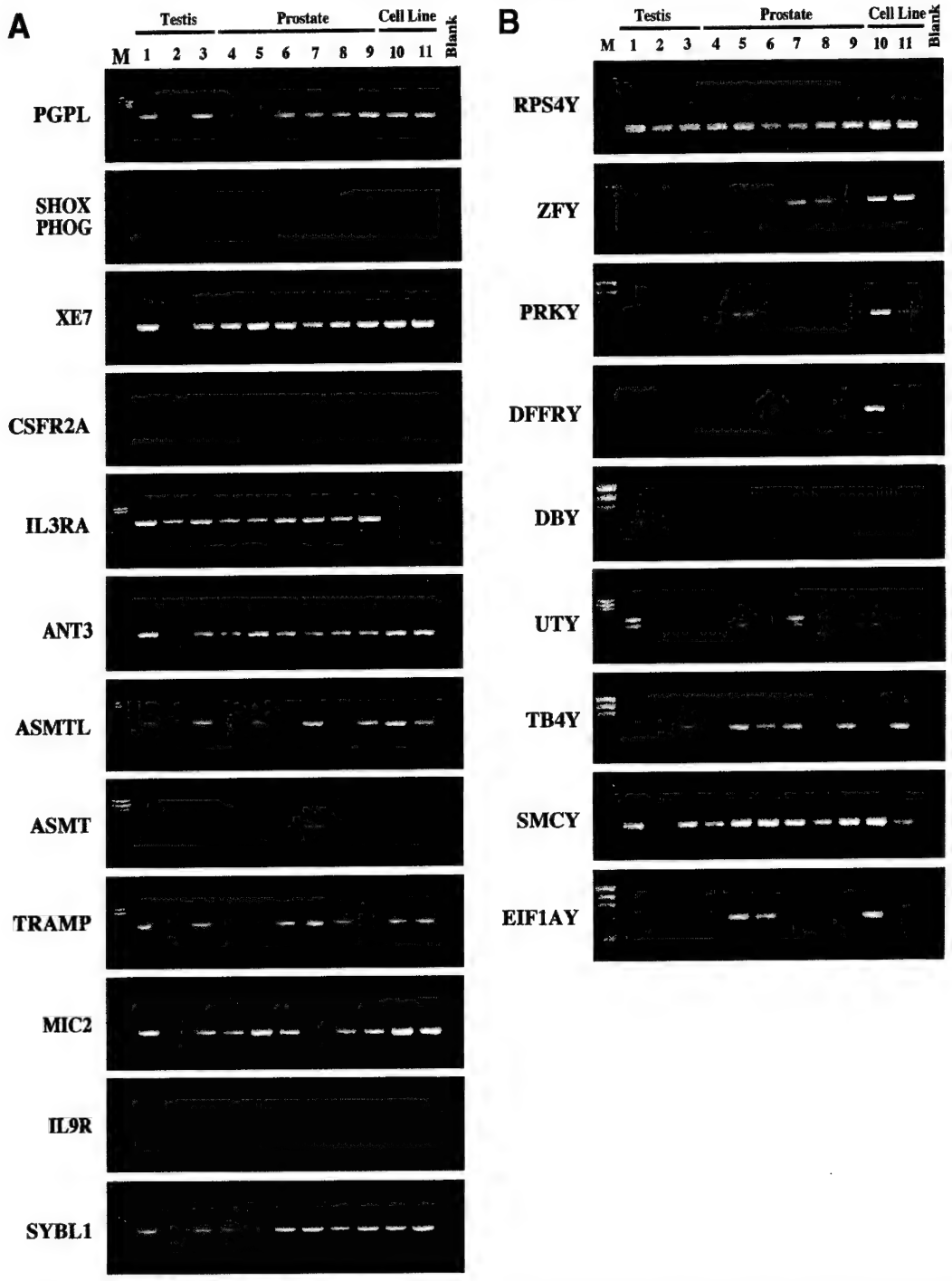

Figure 1. Expression of Y chromosome genes in testis and prostate samples and prostatic cell lines. A. X-Y identical genes on the pseudoautosomal regions. B. X-Y homologous genes on the nonrecombining region of the Y chromosome (NRY). C. Y-specific genes on NRY. D. Reference genes and E. HPRT. The + signs indicate RNA samples treated with reverse transcription, the – signs indicate RNA samples without reverse transcription, before PCR. See Table 2 for samples description and Table 1 and 3 for gene designations and primer information respectively. M = DNA fragments from ΦX174 RF DNA digested with Hae III. The fragments are 1353, 1078, 872, 603, 310, 271–281, 234, 194, 118, 72 bp from top to bottom.

In summary, this initial survey of Y chromosome gene expression in prostate tissues (BPH, BPH + CA, and CA) and prostatic cell lines (androgen responsive and independent) suggested that genes on this chromosome could be divided into several categories. The first category of genes consisted of tissue-specific genes, such as *AMELY*, *SHOX/PHOG*, and *IL9R*. Their expression in prostate tissues was minimal, if detected. Genes for the second category were expressed almost uniformly in most prostate

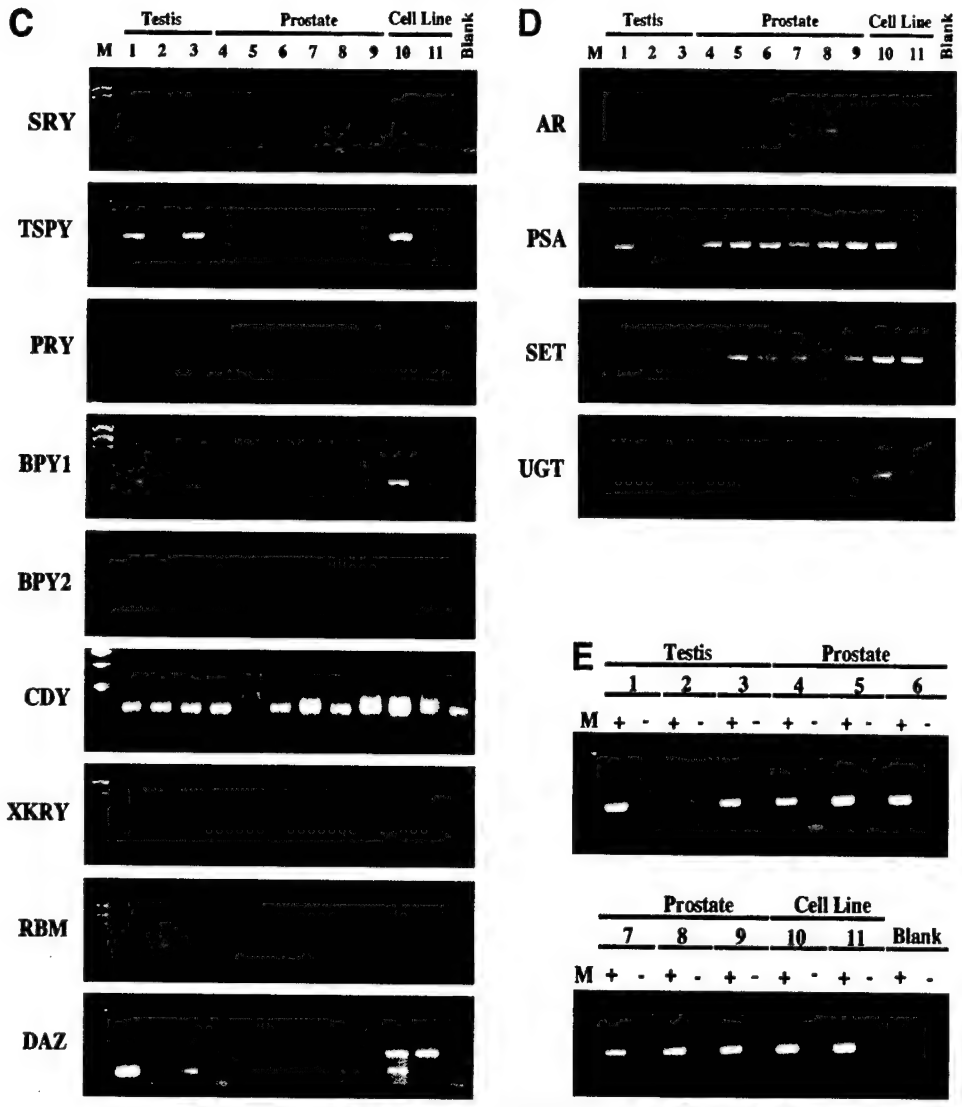

Figure 1. (*Continued*)

tissues (of different pathological classification). They most likely serve essential, and presumably normal, functions in this organ. Members of this category were derived primarily from X-Y identical (PAR), such as *ANT3*, *XE7*, and *MIC2*, and X-Y homologous genes, such as *RPS4Y*, *DBY*, and *SMCY*. The third category of genes consisted of mostly Y-specific and X-Y homologous genes. Their expression levels varied from sample to sample. These genes, particularly the Y-specific ones, are most interesting since their expression patterns may reflect the oncogenic conditions of the prostate samples and cell lines examined.

Androgen Regulation of Y Chromosome Genes

We used the relatively quantitative touchdown RT–PCR technique to evaluate the regulation of Y chromosome genes in the hormonally responsive LNCaP cell line. LNCaP cells were initially cultured in hormone-depleted media. A synthetic androgen, R1881, was then added to the media in different flasks, with concentrations of 10^{-10}, 10^{-9}, and 10^{-8} M. The cells were cultured for an extended period of nine days before they were harvested for total RNA preparation. Numerous studies had demonstrated that similar R1881 treatments stimulate the proliferation of LNCaP cells in a dosage dependent manner [9–11]. The RNA samples from untreated controls and cells treated with R1881 were analyzed with the touchdown RT–PCR procedure by using gene-specific primer sets, as described. Results from this experiment indicated that only a few Y chromosome genes were affected by hormonal treatment of the cells (Figure 2A–D). They include the *IL9R, PGPL, CSFR2A, IL3RA, TSPY, SRY, ZFY, PRY* genes. The steady state transcripts from *TSPY, PGPL, CSFR2A, IL3RA*, and *IL9R* genes increased while those of *SRY, ZFY*, and *PRY*

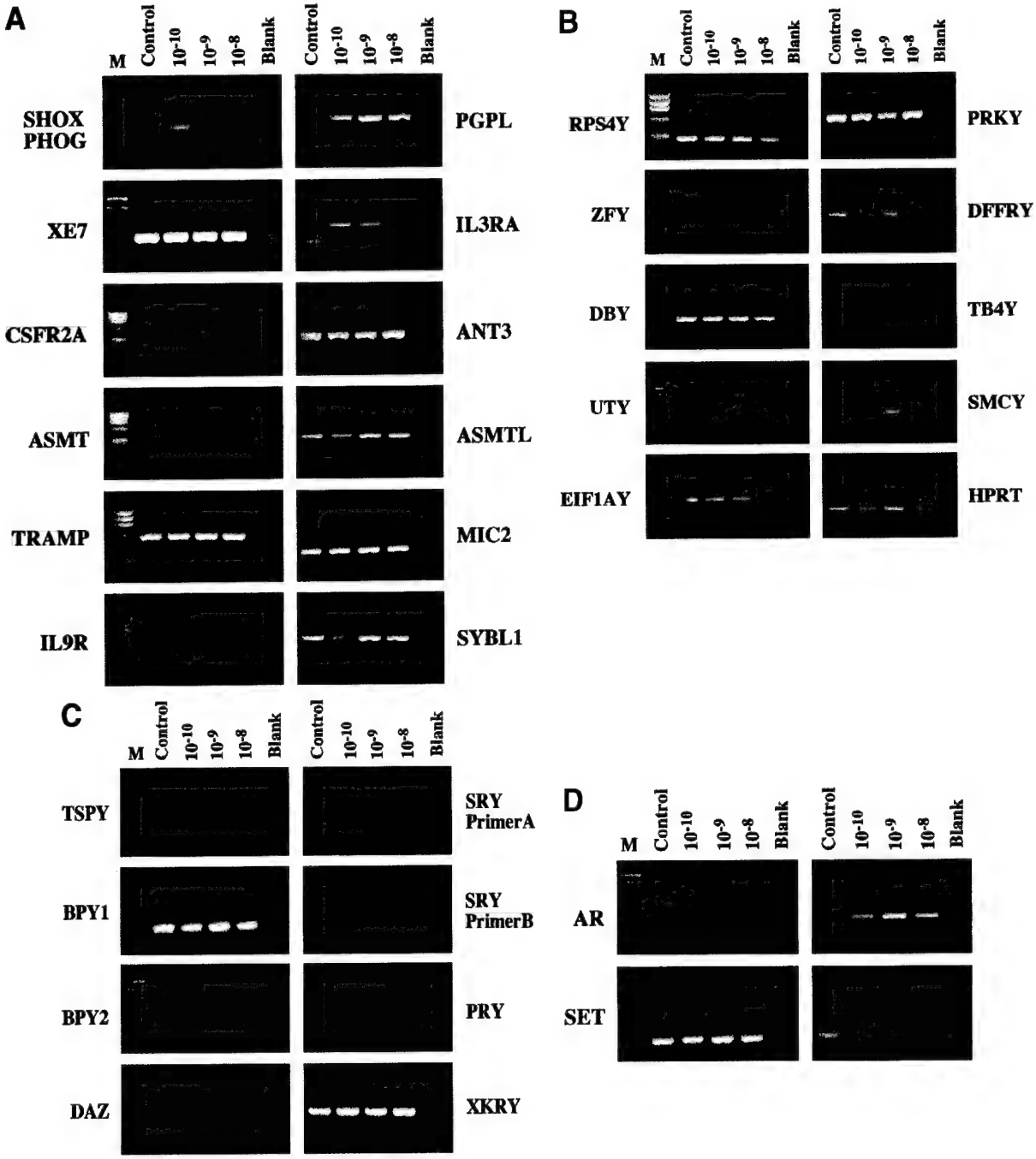

Figure 2. Androgen regulation of Y chromosome gene expression in LNCaP cells, cultured with 0 (control), 10^{-10}, 10^{-9} and 10^{-8} M of synthetic androgen, R1881. A. X-Y identical genes and HPRT control. B. X-Y homologous genes on NRY. C. Y-specific genes on NRY. D. Reference genes. The steady state transcripts from *ZFY, SRY,* and *PRY* on the Y chromosome and *AR* and *UGT* reference genes were down regulated while those for *IL9R, PGPL, CSFR2A, IL3RA, TSPY,* and *PSA* were up regulated upon androgen treatment of LNCaP cells.

decreased in LNCaP cells by R1881 treatments. The down regulation seemed to be extreme for the *SRY* gene. At a small dose of 10^{-10} M of the androgen, the *SRY* product (Primer A) was totally undetectable while those from the other Y gene transcripts were easily observed. To confirm these results, the PCR amplification was repeated with a second set of *SRY* primers (Primer B). Again, PCR products from androgen treated cells were drastically reduced (Figure 2C). Parallel PCR analysis of other Y chromosome genes, e.g., *XKRY, XE7, ANT3, DBY,* and *RPS4Y,* showed uniform amplification of the respective products.

Transcript levels for the five reference genes, *HPRT, AR, PSA, SET,* and *UGT,* were analyzed similarly in the LNCaP cells. The *PSA* gene showed

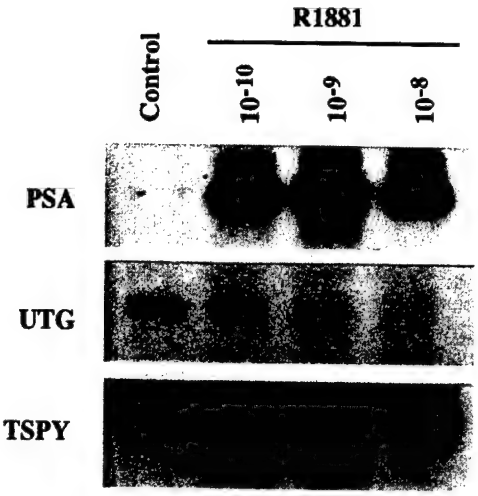

Figure 3. Northern blotting analysis of *PSA*, *UTG*, and *TSPY* transcripts in LNCaP cells treated with the synthetic androgen, R1881. *PSA* and *TSPY* steady state transcripts increased and those for *UGT* decreased under androgen stimulation of LNCaP cells.

a gradual increase while *UGT* and *AR* genes showed gradual decreases of their respective transcripts upon R1881 treatments (Figure 2D). Both *HPRT* and *SET* genes showed no apparent response to androgen regulation. Previously, *UGT* expression had been demonstrated to be down-regulated with androgen treatments in this prostatic cell line [17]. Northern blotting analysis of RNAs from the respective LNCaP cell populations indeed showed increases in steady state transcripts of both *PSA* and *TSPY* genes and a decrease of *UGT* transcripts (Figure 3). Although there was a general correlation between the RT–PCR and northern hybridization results, the relative amounts of transcripts for these genes detected by northern blotting were not as linear as those detected by RT–PCR (e.g., Figure 2). These differences may be attributed to the sensitivity of the respective methods.

DISCUSSION

Prostatic carcinogenesis is a multi-step process mediated by many genetic and environmental components. One or a few of the genetic components could be derived from genes on the Y chromosome. Unfortunately, the assessment on the contribution of this chromosome to the etiology of prostate cancer has been difficult, although the Y chromosome is absolutely essential for male development, including organogenesis of the testis and the prostate gland. There are two main reasons that may be attributed to this problem. First, major portion of this chromosome does not pair with the X chromosome and does not go through any meiotic recombination, thereby making any linkage analysis to be extremely difficult, if not impossible. Consequently, most of the genetic linkage studies on families with hereditary prostate cancer had excluded the Y chromosome in their analyses [48–51]. Second, both classical cytogenetic and/or chromosome painting studies documenting either a gain or a loss of this chromosome in tumor cells derived from prostate cancer [52–55] were relatively crude and did not provide any specific information on the individual Y chromosome genes in the samples analyzed. These results contributed to the controversy regarding the role of this chromosome in prostate cancer. Recently, Tricoli [56] argued that many studies utilizing samples from either short-term cultures and/or tissue sections might give rise to clonal cell selection and nuclear truncation, respectively. He suggested that the Y chromosome was present in most of the prostate cancer specimens. To demonstrate his point, Tricoli [56] had studied the status of the Y chromosome on touch preparations from 42 fresh prostate tumors by using chromosome painting technique. The touch preparation allowed him to transfer intact epithelial tumor cells directly onto a slide without paraffin or frozen sectioning procedures. Results from Tricoli's study demonstrated a gain of the Y chromosome in a single sample and normal number of Y chromosome in the remaining 41 samples, suggesting that loss of the Y chromosome is an infrequent event in prostate cancer. This study, if confirmed independently, supports Tricoli's postulation that this male-specific chromosome is present in the prostate during its oncogenic process. Further, there is genetic evidence suggesting that first-degree males (i.e., father, son, or brother) of a prostate cancer patient who have the same Y chromosome might have a higher probability of getting the disease than those without a first-degree male suffering from prostate cancer [45–47]. These observations, taken together, signify that the genes on this male chromosome might play some roles in prostatic carcinogenesis. Indeed, a cancer locus, termed gonadoblastoma on the Y chromosome or GBY, has long been mapped on the human Y chromosome [57,58]. The GBY locus harbors a gene(s) that predisposes the dysgenetic gonads of XY females to develop gonadoblastoma at a high (>30%) frequency. Significantly, recent isolation of most of the genes residing on the human Y chromosome [1–4] has presented a panel of candidate genes that may play some roles in cell growth, cell cycle regulation, and signal transduction (Table 1). Mutations and/or aberrant expression of one or a few of these genes may contribute to the oncogenic processes not only in gonadoblastoma but also in male-specific organs, including the testis and the prostate gland.

Previous studies had demonstrated the expression of both *SRY* and *ZFY* in over 60% of 30 BPH and prostate cancer samples [7,8]. The present study confirms a similar level (four of six samples) of expression for both genes among the prostatic samples analyzed. In addition, we have further

demonstrated that these two genes, as well as *PRY*, were down-regulated in LNCaP cells upon androgen treatments. *SRY* has been demonstrated to be the gene for *TDF* responsible for switching a male development during embryogenesis [5]. Although the exact mechanism(s) by which *SRY* mediates sex determination is uncertain, a repressor mode of action has been proposed [23]. It's function in adult tissues is still uncertain. If *SRY* serves as a repressor for cell growth or proliferation, its down-regulation would be needed for cellular proliferation events. *ZFY* encodes a transcription factor with DNA-binding zinc fingers [24]. It has a homolog, *ZFX*, on the X chromosome. Both *ZFY* and *ZFX* genes have distinct expression patterns different from each other [25]. The *Zfx* gene in the mouse may play a role in germ cell development since male and female mice harboring a null *Zfx* gene on their X chromosome showed reduced numbers of germ cells in their respective sex organs [26]. *PRY* is a recently isolated repeat gene on the human Y chromosome. It encodes a protein homologous to the protein tyrosine phosphatase, *PTP-BL* [2,27]. Significantly, protein kinases and phosphatases have been postulated to be regulators and signal transducers in cell growth and proliferation [28]. The down-regulation of *PRY* suggests a possible involvement of this putative tyrosine phosphatase in androgen stimulation of LNCaP cells.

Androgen plays an essential role in the development and differentiation of prostate in embryos and prostate cancer pathogenesis in adults. Androgen regulates the expression of genes via the androgen receptor activation pathway [18–22]. Many of these androgen-regulated genes are growth factors and extracellular matrix components that act specifically on the epithelial cells and stimulate their proliferation and differentiation. It has been argued that a similar, if not the same, set of genes may be involved in the regulation of normal development of the embryonic prostate and in oncogenesis of the adult prostate. Although the Y chromosome has been demonstrated to be critical in switching on testis determination and differentiation during embryogenesis [5], its role in prostate development has not been clearly defined. Thus, the identification of androgen-regulated expression of any of the Y chromosome genes would suggest potential candidates important for both prostate organogenesis and oncogenesis.

The present study indeed has identified several PAR genes, *PGPL, CSFR2A, IL3RA, IL9R*, and the Y-specific gene, *TSPY*, to be up-regulated by androgen treatments in LNCaP cells. The *PGPL* gene encodes a putative small GTP-binding protein on the tip of the PAR1 [3]. Numerous studies have demonstrated that small GTPases, such as the Rho family, participate in various cytoskeletal organization and signaling processes and Ras-mediated cell transformation [32–34]. If the *PGPL* product indeed can function as a GTPase, it may potentially play a similar role in cell signaling and transformation. The up-regulation of the three cytokine receptor genes, *IL9R, CSFR2A*, and *IL3RA*, suggests that cytokines and their receptors may play a role(s) in androgen-stimulated proliferation in this prostatic cell line. *IL9R* encodes the receptor for IL-9 [29] while *CSFR2A* and *IL3RA* encode the α subunits of receptors for the granulocyte-macrophage colony-stimulating factor (GM-CSF) and the IL-3, respectively [35–42]. Each α subunit exhibits specific affinity to the respective growth factor but requires heterodimerization with the β subunit to form a fully functional receptor [35–38]. Recent studies have demonstrated that GM-CSF and its receptors are expressed in prostatic cell lines LNCaP, PC3, and DU-145 [39–41]. Further recombinant human GM-CSF stimulates proliferation of the LNCaP cells in vitro [40], suggesting that prostatic cells are responsive to this hematopoietic factor. GM-CSF mediates the proliferative function through its receptor that induces phosphorylation events in the LNCaP cells [40]. Immunostaining experiments localized both α and β subunits of the GM-CSF receptor on the epithelial cells of normal prostate, BPH, and prostatic carcinoma. Both subunits are expressed at low level in normal human prostatic tissue, at substantial level in BPH, and at prominent level in prostatic carcinoma [40]. Hence, GM-CSF and its receptor may play a role in prostatic cell proliferation and oncogenesis. The cytokines, IL3, IL9, and their receptors have been demonstrated to play a role(s) in T cell and other hematologic malignancies [42], their roles in prostatic cells are still unknown.

Using a mRNA differential display technique, Shen and colleagues [7] had recently identified *MIC2* to be a gene up-regulated by androgen treatment of LNCaP cells. *MIC2* expression was responsive to androgen stimulation in the parental LNCaP line, but not in sublines with higher metastatic potential. Immunostaining experiments detected the *MIC2* encoded product, E2, in tissues from patients with primary prostate cancer, but only sporadically in benign prostatic hyperplasia tissues. Our results confirmed that *MIC2* was expressed in mostly prostatic cancer and BPH tissues. However, its steady state RNA level was relatively uniform in LNCaP cells treated with various dosages of R1881. It is uncertain if the differences in our observations and those of Shen and colleagues [7] reflect the methodology and/or cell lines used in these studies.

The *TSPY* gene constitutes the most interesting gene on this chromosome whose aberrant and androgen-responsive expression may be involved in prostatic cell proliferation and oncogenesis in the prostate gland. *TSPY* is a repeat gene with about 20–40 copies on the human Y chromosome [see review, 30]. It is primarily located in the deletion interval 3

on Yp11.2, within a small region where the gonadoblastoma locus has been mapped on this chromosome [57–58]. Hence, *TSPY* is a candidate for *GBY* and whose expression has been demonstrated in gonadoblastoma tissues [30,58, Lau et al., unpublished observations]. TSPY protein shares extensive homology with that of the *SET* oncogene, involved in an intra-chromosomal translocation on chromosome 9 of a patient with undifferentiated acute leukemia [16]. *SET* is a member of a family of cyclin B-binding protein genes that includes the nucleosome assembly protein (*NAP*-1). Some members of this protein family are involved in cell cycle regulation. The *TSPY* gene is expressed normally in spermatogonial cells of the testis and has been hypothesized to direct these germ stem cells to proliferate and/or to enter meiosis [31]. Recently, aberrant expression of *TSPY* has been documented in testicular cancer samples, including germ seminoma and carcinoma-in-situ [30,31]. Our study showed that its expression in LNCaP cells could be stimulated by androgen treatments, suggesting that *TSPY* may be associated with the proliferative activities of these cells under such conditions.

The present survey of Y chromosome gene expression in prostate cancer and BPH samples has highlighted this portion of the human genome that is important for the normal differentiation and/or physiology of male-specific organs. Although there was no clear-cut pattern of Y gene expression specifically related to the degrees of malignancy in our specimens, a small number of genes, such as *TSPY, SRY, PRY, ZFY*, and the cytokine receptors, have been identified to play a potential role(s) in or to be influenced by oncogenesis in the prostate. These results, hence, form the basis for further studies using advanced techniques, such as in situ mRNA hybridization, immunohistochemistry, microdissection and RNA analysis, on clinically defined sets of specimens. Such investigations focusing on individual Y chromosome genes may provide significant insights into the likely contribution of this male-specific chromosome to the initiation and progression of prostate cancer.

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Cytogenetics and Cell Genetics

Expression of a candidate gene for the gonadoblastoma locus in gonadoblastoma and testicular seminoma

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Dedicated to Professor Dr. Ulrich Wolf on the occasion of his retirement.

Abstract. The gonadoblastoma locus on the Y chromosome (GBY) predisposes the dysgenetic gonads of XY females to develop in situ tumors. It has been mapped to a critical interval on the short arm and adjacent centromeric region on the Y chromosome. Currently there are five functional genes identified on the GBY critical region, thereby providing likely candidates for this cancer predisposition locus. To evaluate the candidacy of one of these five genes, testis-specific protein Y-encoded (TSPY), as the gene for GBY, expression patterns of TSPY in four gonadoblastoma from three patients were analyzed by immunohistochemistry using a TSPY specific antibody. Results from this study showed that TSPY was preferentially expressed in tumor germ cells of all gonadoblastoma specimens. Additional study on two cases of testicular seminoma demonstrated that TSPY was also abundantly expressed in all stages of these germ cell tumors. The present observations suggest that TSPY may either be involved in the oncogenesis of or be a useful marker for both types of germ cell tumors.

Gonadoblastoma is a rare tumor that arises mostly in the dysgenetic gonads of phenotypic females who harbor some Y chromosome materials in their genome (Page, 1987). The tumor is composed of aggregates of primordial germ cells and sex cord elements resembling immature Sertoli and granulosa cells. These aggregates are surrounded by luteinized ovarian type stroma that may include Leydig or lutein-type cells (Scully, 1953, 1970). Gonadoblastoma has been considered to be an in situ germ cell malignancy from which invasive germ cell tumors can develop (Skakkebaek et al., 1987; Jorgensen et al., 1997; Looijenga and Oosterhuis, 1999; Heerbst et al., 1999).

The prevalence of gonadoblastoma among XY females had led David Page (1987) to hypothesize the presence of a locus, gonadoblastoma locus on the Y chromosome (GBY), that predisposes the dysgenetic gonads of these sex-reversed individuals to develop such in situ tumors. Page further predicted that the gene(s) encoded by the GBY locus has a normal function in the testis and acts as an oncogene only in the dysgenetic gonad. Using a panel of DNAs from XY females with gonadoblastoma, Page had initially mapped the GBY locus to deletion interval 3 on the short arm and intervals 4B-7 on the long arm of the Y chromosome. Additional studies further sublocalized this locus to a small region consisting of ~ 1–2 Mb of DNA in deletion intervals 3E–3G proximal to and 4B at the centromere and possibly 5E, a proximal interval on the long arm (Salo et al., 1995; Tsuchiya et al., 1995). Among the genes so far isolated from the human Y chromosome (Vogt et al., 1997; Lahn and Page, 1997; Lau and Zhang, 2000), there are five genes residing on this small region: amelogenin Y (AMELY), RNA binding

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motif Y (RBMY), protein kinase Y (PRKY), protein tyrosine phosphatase PTP-BL related Y (PRY), and testis-specific protein Y-encoded (TSPY). Hence, they are candidates for GBY. AMELY encodes an enamel protein in the tooth buds (Salido et al., 1992). RBMY is a repeated gene with a majority of its functional members residing at interval 6 on the long arm, outside the GBY critical region (Cooke, 1999). It expresses a protein with RNA binding motif in the nuclei of male germ cells. PRKY is a single copy gene coding for a putative cAMP-dependent serine/threonine protein kinase (Schiebel et al., 1997). Both RMBY and PRKY have a homologous gene, RBMX and PRKX respectively, on the X chromosome (Schiebel et al., 1997; Delbridge et al., 1999). PRY is a recently isolated repeated gene family coding for a protein related to the PTP-BL tyrosine phosphatase (Lahn and Page, 1997). Some copies of PRY are present outside the GBY region. TSPY is a repeated gene whose functional members are primarily located in two clusters, TSPYA and TSPYB, within interval 3 (Zhang et al., 1992; Conrad et al., 1996; Vogt et al., 1997) on the short arm and as a single-copy on the proximal region of the long arm (Ratti et al., 2000). TSPY shares tight homology to a family of cyclin B binding proteins, such as the SET oncoprotein and the nucleosome assembly protein (NAP-1) (Tsuchiya et al., 1995; Schnieders et al., 1996), and has been postulated to play a role in directing the spermatogonial cells to enter meiosis (Schnieders et al., 1996; Vogel et al., 1998). Other cyclin B binding proteins have been demonstrated to be involved in the mitotic process, cell proliferation and/or carcinogenesis (von Lindern et al., 1992; Adachi et al., 1994; Altman and Kellogg, 1997; Carlson et al., 1998; Shin et al., 1999). Hence, aberrant or inappropriate expression of TSPY in dysgenetic gonads may play a role in the etiology of gonadoblastoma.

To evaluate the candidacy of TSPY as the gene(s) for GBY, we have performed detailed expression analysis of TSPY in gonadoblastoma and testicular seminoma, or germ cell tumor, using immunohistochemical techniques. Our results demonstrate that TSPY is preferentially expressed in the germ cells of the tumor aggregates in gonadoblastoma and tumor cells at different stages of testicular seminoma. Its expression pattern is very similar to those of cyclin B1 and another cell proliferative marker, the proliferating cell nuclear antigen (PCNA). These findings, hence, support the postulation that TSPY is a significant candidate for GBY, and suggest a possible role of TSPY in the multi-step carcinogenesis of testicular seminoma.

Materials and methods

Patients

Tissue sections were obtained from archival formalin-fixed and paraffin-embedded tumor specimens. All three gonadoblastoma patients had previously been described (Iezzoni et al., 1997; Hussong et al., 1997). At the time of biopsies, Patient #1 was a 15-year old phenotypic female with a unilateral gonadoblastoma at the left gonad. Patient #2 was a 20-year old phenotypic female with bilateral gonadoblastoma. Both patients #1 and 2 have a 45,X/46,XY mosaic karyotype. Chromosome painting analysis on tissue sections of these tumors showed that most tumor cells harbored a Y chromosome while the stroma showed reduced numbers of cells harboring this chromosome (Iezzoni et al., 1997). Patient #3 was a 15-year old phenotypic female with a 46,XY karyotype. She developed a tumor mass on the left and a streak gonad on the right (Hussong et al., 1997).

Two testicular seminoma specimens were obtained from archival samples at the Anatomic Pathology Section, VA Medical Center, San Francisco. At the time of orchiectomy, Patient #1 was a 49-year old male with a tumor mass confined only to the left testicle. Pathological examination revealed a classical seminoma. Patient #2 was a 49-year old male with an advanced and mixed germ cell tumor composed of seminoma, embryonal carcinoma and yolk sac tumor.

Generation of a specific antibody against TSPY

The entire open reading frame of the human TSPY cDNA (Zhang et al., 1992) was subcloned in-frame in the *Eco*R1 site of the expression vector, pAR(ΔR1) (Blanar and Rutter, 1992), a derivative of the pET3a vector. Recombinant TSPY protein was synthesized in bacterial host, BL21DE3 (pLysS) and purified by preparative SDS-PAGE from total lysates of induced bacterial culture. A polyclonal antiserum was generated by repeat immunizations of a New Zealand white rabbit using the service of a commercial vendor (Vancouver Biotechnology, Vancouver, Canada). The specificity of the antiserum was initially assayed by Western blotting against recombinant TSPY protein. The specificity of this antibody to TSPY was further confirmed by both Western blotting and immunocytochemical staining of HeLa cells expressing at high levels a transfected human TSPY gene (Lau, unpublished observations). A polyclonal antibody against the proliferative cell nuclear antigen (PCNA) was purchased from Dako Laboratory, Inc. (Carpinteria, CA). A polyclonal antibody against the human cyclin B1 (synthesized with a bacculuvirus vector in insect cells) was a gift from Catherine Takizawa and David Morgan, Department of Physiology, UCSF. Both antibodies had previously been demonstrated to be specific for the respective antigens in Western blotting and immunostaining studies (Jin et al., 1998; Takizawa et al., 1999; Kömüves et al., 1999).

Immunohistochemistry of tumor tissue sections

Five-micron sections were obtained from archival materials according to established procedure. Immunohistochemical staining was performed as previously described (Kömüves et al., 1999). Heat-induced antigen retrieval pretreatment was utilized in procedures with PCNA and cyclin B1 antibodies. Immunostaining was conducted in a Tris buffer, pH 7.6, containing 4% of bovine serum albumin, 1% gelatin, 0.1% Tween 20, and 500 mM NaCl. The primary antisera were used at 1:500 to 1:1000 dilution ratios. The binding of the primary antibody was detected by reaction with affinity-purified biotinylated goat anti-rabbit IgG, and visualized by either ABC-peroxidase or ABC-alkaline phosphatase reagents. Commercial substrate kits (Vector Laboratories, Burlingame, CA) were used for the enzymatic detections. For the brown and brick-red signals, the DAB and VECTOR NovaRED kits were used respectively with the ABC-peroxidase reagents. For the red signal, the VECTOR RED kit was used with the ABC-alkaline phosphatase reagents. All signals were dependent on the bindings of the respective primary antibodies and were independent of the substrate kits used. After the immunostaining, the sections were counter-stained with hematoxylin. Omitting the primary antibody in the procedure resulted in no signals. Preabsorption of the TSPY antiserum with excess recombinant TSPY protein abolished the staining. The sections were examined and recorded with a Zeiss Axiophot microscope.

Results

Preferential expression of TSPY in tumor germ cells of gonadoblastoma

Histological analysis of the gonadoblastoma specimens from all three patients showed characteristic aggregates of primordial germ cells and sex chord elements. Immunostaining of tissue sections of all four tumors (two patients with unilateral and one patient with bilateral tumors) showed positive staining of cancerous germ cells within these tumor aggregates of all three patients (e.g. Fig. 1A). In particular, the tumor of patient #3 was organized in smaller aggregates with less sex chord elements (Fig. 1B, C), partially resembling the morphology of some forms of germ cell tumors in the testis (e.g. Fig. 1L, M).

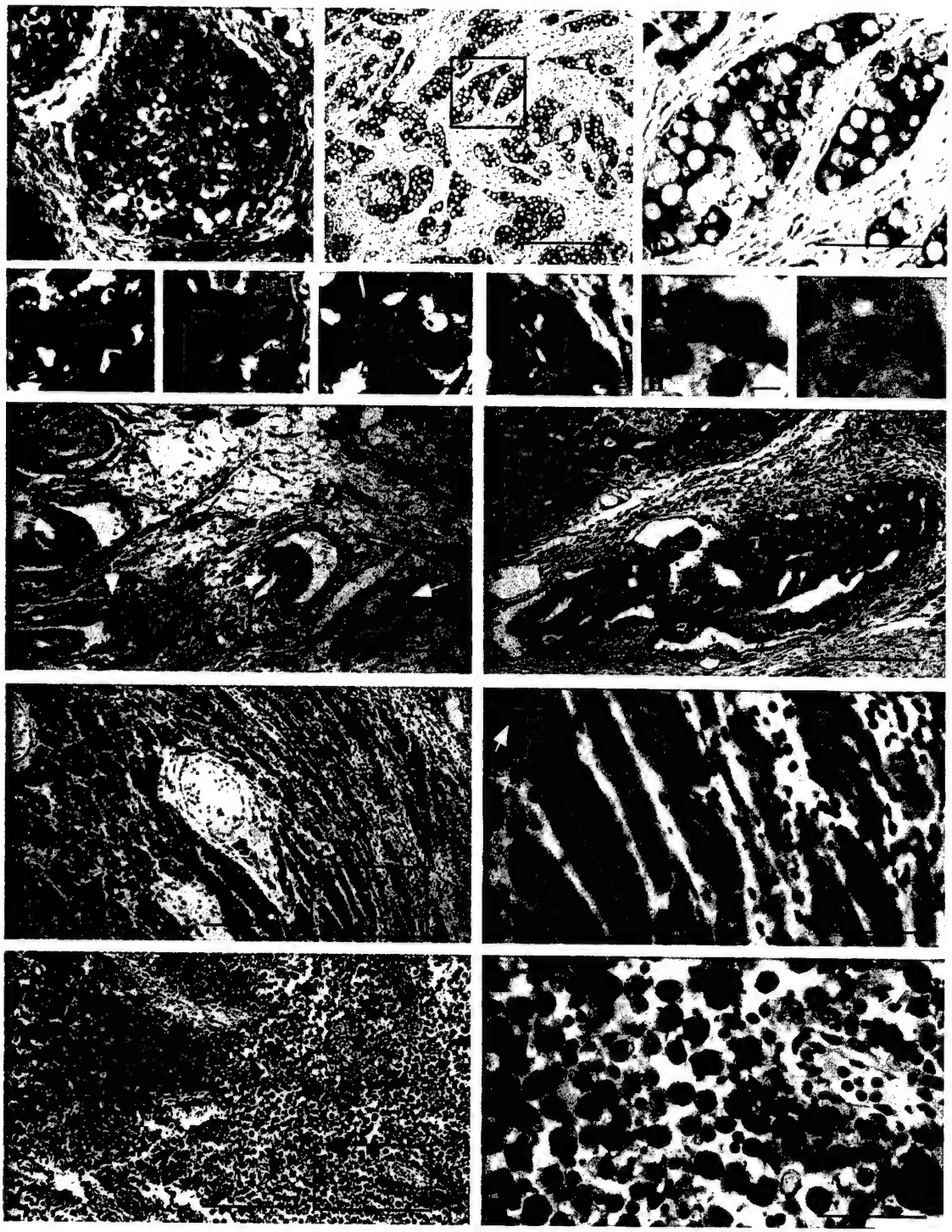

Fig. 1. Immunohistochemical localization of TSPY in gonadoblastoma and testicular seminoma. (**A**) TSPY was primarily located in the germ cells of tumor aggregates of all three gonadoblastoma patients. This figure illustrates an example of immunostaining on sections from Patient #1. (**B**) Patient #3 harbored a gonadoblastoma with less organized aggregates. (**C**) An enlargement of boxed area in **B**, showing prominent cytoplasmic locations of the TSPY protein. (**D–I**) Examples of mitotic cells within the gonadoblastoma from patient #3. Cells in **D–G** were stained with TSPY antibody; **H** with PCNA antibody and **I** a control without primary antibody reaction. (**J–O**) Immunostaining of TSPY on tumor sections from seminoma at early (**J**), intermediate (**K**) and late (**L–O**) stages of the testicular cancer. Yellow arrows in **J** point to possible localized tumor growth areas. Blue arrow in **L** indicates a tubule being abandoned by the invasive growth of the tumor (boxed area enlarged in **M**). **N** Shows an advanced tumor area consisting of large mass of tumor cells and highly undifferentiated and loosely associated embryonal cells (boxed area enlarged in **O**). White arrows in **M** and **O** point to mitotic tumor cells. Positive signals are brick red in **A**; chocolate in **B** and **C**; brown in **D–H**; and red in **J–O**. Bars indicate 50 μm in **A**, **C**, **M** and **O**; 200 μm in **B**, **J**, **L**, and **N**; and 10 μm in **D–I**.

The TSPY protein was prominently localized primarily in the cytoplasm of these large cells (Fig. 1C). In all cases, the sex cord elements and the stroma showed very little reactive signals (Fig. 1A, B). Mitotic cells were readily observed on specimens from this patient (Fig. 1D–I). Omission of the primary antibody or pre-absorption of the antiserum with excess recombinant TSPY protein abolished or greatly reduced the reactive staining of the germ cells in these procedures (e.g. Fig. 1I). Analysis of parallel sections with PCNA and cyclin B1 antibodies showed similar staining patterns as those with the TSPY antibody (data not shown). The signals were primarily located on the nuclei for PCNA while those for cyclin B1 seemed to be associated with both nuclei and cytoplasm of the germ cells. The general staining patterns indicated that TSPY, PCNA and cyclin B1 were co-expressed in the same tumor cells.

TSPY expression in the tumor cells at various stages of testicular seminoma

It has been argued that gonadoblastoma is a precursor form of more aggressive germ cell tumors (Skakkebaek et al., 1987; Jorgensen et al., 1997; Looijenga and Oosterhuis, 1999; Herbst et al., 1999). If TSPY is indeed the candidate for GBY, it would potentially participate in the oncogenic process of other germ cell tumors. Previously, Schneiders and colleagues (1996) had indeed demonstrated an up-regulation of TSPY expression in in-situ carcinoma of the testis, a presumed precursor of germ cell tumors or seminoma. To address the question of whether TSPY may also play a role in more advanced testicular cancer, we have extended our study to include two cases of seminoma using the same immunohistochemical staining technique and specific antibodies against TSPY, PCNA and cyclin B1. They, together, showed various morphological forms potentially representing the different oncogenic stages of these tumors. At the early stages, spermatogenesis might have ceased, thereby depleting normal meiotic cells within the seminiferous tubules. In-situ carcinoma could also occur as a precursor during this initial carcinogenic period (Schnieders et al., 1996). The germ cell-depleted and epithelium-like tubules consisted of a single-layer of cells that might develop into localized multi-layer tumors at various peripheral segments (Fig. 1J, yellow arrows). This transformation progressed until most of the epithelia were lined with multiple layers of tumor germ cells (Fig. 1K). Invasive aggregates of cancerous germ cells eventually evolved from such tubular tumors, abandoning the original tubules (Fig. 1L, blue arrow). In the advanced stages, these aggregates could form a large tumor mass covering a sizable portion of the testis (Fig. 1L, N). Occasionally highly undifferentiated and loosely associated embryonal cells were observed in the late stages of these tumors (Fig. 1O). Presumably, these single tumor cells might have acquired some metastatic properties/potential. Significant expression of the TSPY protein was detected in the tumor germ cells at all stages of these testicular cancers (Fig. 1J–O). Similar to the expression pattern in gonadoblastoma, TSPY was primarily located on the cytoplasm of the tumor cells (Fig. 1M, O). Occasionally, nuclear locations of TSPY were also detected in a few cells. Under each microscopic view, a significant number of mitotic cells could easily be identified (white arrows, Fig. 1M, O). Similar to those in gonadoblastoma, most mitotic cells were stained positively with the TSPY antibody. TSPY expression was at a reduced or insignificant level in the interstitial regions of these testicular cancers.

Immunohistochemical staining of parallel tissue sections with PCNA and cyclin B1 antisera demonstrated similar expression patterns as that of TSPY for both antigens in testicular seminoma (data not shown). Again, PCNA showed a mostly nuclear location while cyclin B1 showed a nuclear and cytoplasmic staining pattern. These observations suggested a possible co-expression of these three proteins in the same tumor germ cells.

Discussion

The mapping of the GBY locus within a small region of the human Y chromosome suggests the existence of a proto-oncogene on this chromosome that predisposes the dysgenetic gonads of XY females to malignancy (Page, 1987; Salo et al., 1995; Tsuchiya et al., 1995). The identification of candidate genes for GBY will not only provide information on the molecular etiology of gonadoblastoma but will also shed light on the contribution of this chromosome to the carcinogenic processes of other male-specific cancers, such as testicular seminoma, germ cell tumors and prostate cancer (Looijenga and Oosterhuis, 1999; Lau, 1999; Lau and Zhang, 2000). Recent studies on TSPY have positioned it to be the most viable candidate for GBY within the critical region on the Y chromosome. First, it is present in the DNAs of gonadoblastoma patients (Salo et al., 1995; Tsuchiya et al., 1995). Its transcripts had been detected by RT-PCR technique in the corresponding tumor tissues (Tsuchiya et al., 1995) and recently by immunostaining in a single case of gonadoblastoma (Hildenbrand et al., 1999). Second, immunostaining studies had demonstrated its expression in spermatogonia in normal testis (Schnieders et al., 1996), suggesting that TSPY may serve a normal function of directing the spermatogonial cells to enter meiosis, a condition for the GBY gene(s) initially postulated by David Page (1987). Third, the TSPY protein is highly homologous to a family of cyclin B binding proteins, including NAP-1 and SET oncoprotein (Tsuchiya et al., 1995; Schnieders et al., 1996), suggesting that it may bind to this mitotic cyclin and be involved in cell cycle regulation and/or cell proliferation (Shin et al., 1999). Results from the present study demonstrate that TSPY is preferentially expressed in the proliferating germ cells within the tumor aggregates in all four samples from three gonadoblastoma patients, further supporting the candidacy of TSPY for GBY.

Although TSPY protein has previously been detected in some forms of testicular tumors, including in-situ carcinoma (Schnieders et al., 1996), our study on the seminoma specimens has clearly demonstrated the high levels of expression of this GBY candidate gene in all stages of these advanced germ cell tumors. Hence, these results, together with those observed by others (Schnieders et al., 1996), establish a direct relationship between the aberrant TSPY expression and the oncogenic process of testicular cancer. Numerous studies have demonstrated that cyclin B binding proteins, such as SET, are either involved in oncogenesis of acute leukemia, Wilm's tumor or modulation

of cell proliferation (von Lindern et al., 1992; Adachi et al., 1994; Carlson et al., 1998; Shin et al., 1999). Although the interactions between TSPY and cyclin B have yet to be demonstrated experimentally, the co-expression of these two molecules on the same tumor cells has raised the possibility that they may indeed interact in vivo. The present study has provided evidence supporting the hypothesis that aberrant expression of TSPY may lead to abnormal cell proliferation and tumor formation (Lau, 1999). Hence, TSPY is not only a key candidate for GBY but may also contribute to the oncogenesis of testicular seminoma.

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Note added in proof

Recently Stuppia et al. (2000) have demonstrated by cloning and sequence analysis that the PRY gene spans 25 kb in size and contains 5 exons. The functional copies of PRY are located in interval 6 on Yq while those on Yp retain only exon 1 and 2 and are likely non-functional.

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Expression pattern of a gonadoblastoma candidate gene suggests a role of the Y chromosome in prostate cancer

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Abstract. The contribution of specific genes on the Y chromosome in the etiology of prostate cancer has been undefined. Genetic mapping studies have identified a gonadoblastoma locus on the human Y chromosome (GBY) that predisposes the dysgenetic gonads of XY sex-reversed patients to tumorigenesis. Recently a candidate gene, the testis-specific protein Y-encoded (TSPY) that resides on the GBY critical region, has been demonstrated to express preferentially in tumor cells in gonadoblastoma and testicular germ cell tumors. TSPY shares high homology to a family of cyclin B binding proteins and has been considered to possibly play a role in cell cycle regulation or cell division. To address the possible involvement of the TSPY gene in prostate cancer, both in situ mRNA hybridization and immunohistochemistry techniques were used to study the expression of this putative GBY gene in prostate specimens. Our results demonstrated that TSPY was expressed at low levels in normal epithelial cells and benign prostatic hyperplasia (BPH), but at elevated levels in tumor cells of prostate cancers at various degrees of malignancy. Sequence analysis of RT-PCR products obtained from both prostatic and testicular tissues using specific primers flanking the open reading frame of the TSPY mRNA revealed a complex pattern of RNA processing of the TSPY transcripts involving cryptic intron splicing and/or intron skipping. The variant transcripts encode a variety of polymorphic isoforms or shortened versions of the TSPY protein, some of which might possess different biochemical and/or functional properties. The abbreviated transcripts were more abundant in prostatic cancer tissues than the testicular ones. Although the exact nature of such variant TSPY transcripts and proteins is still unclear, their differential expression suggests that the TSPY gene may also be involved in the multi-step prostatic oncogenesis besides its putative role in gonadoblastoma and testicular seminoma.

Failure in testis determination in the presence of the whole or a portion of the Y chromosome predisposes XY sex-reversed individuals to gonadoblastoma development early in life (Scully, 1953, 1970). In 1987, David Page proposed that there is a locus on the Y chromosome, termed gonadoblastoma on the Y chromosome or GBY, that harbors a gene(s) that promotes tumor formation in the dysgenetic gonads of XY sex-reversed females and females with 45,X/46,XY mosaic karyotype (Page, 1987; Lau, 1999). Deletion mapping had localized this locus to small (~1–2 Mb) regions on the short and long arms proximal to the centromere of the Y chromosome. Among the genes located on this critical region for GBY, the Y-specific repeated gene, the testis-specific protein Y-encoded (TSPY) (Zhang et al., 1992; Schnieders et al., 1996) is one of the most significant candidates for this oncogenic or tumor-promoting locus. First, most of the functional copies of the TSPY gene are located in the GBY critical region (Salo et al., 1995; Tsuchiya et al., 1995; Vogt et al., 1997; Stuppia et al., 2000; Rottger et al., 2002; Skaletsky et al., 2003). Second, expression analyses clearly demonstrated high levels of TSPY expression in gonadoblastoma tissues (Hindenbrand et al., 1999; Lau et al., 2000). Additional experiments also detected similarly high levels of TSPY expres-

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sion in the tumor cells of testicular seminoma and carcinoma-in-situ (Schnieders et al., 1996; Lau et al., 2000). Third, TSPY encodes a putative cyclin B binding protein that may be involved in regulation of cell proliferation. These data, taken together, suggest that TSPY may play an oncogenic or cancer promoting role in gonadoblastoma and testicular cancer.

To evaluate the role of the male-specific Y chromosome in prostatic oncogenesis, we had independently examined the expression of 31 Y chromosome genes on a panel of prostate samples diagnosed with benign prostatic hyperplasia (BPH), low and/or high grade carcinoma, and established prostatic cell lines (Lau and Zhang, 2000). This study identified several Y chromosome genes that were heterogeneously and/or differentially expressed among the prostatic samples and/or cell lines. In particular, TSPY was preferentially expressed in prostatic cancer samples. Its expression in the prostatic cancer cell line, LNCaP, was responsive to androgen induction, suggesting that it may be involved in this hormonally sensitive cancer. These findings are significant because as a candidate for GBY, TSPY could potentially play additional roles in the etiologies of other male-specific cancers, such as testis and prostate cancers. To explore this possibility, we have performed detailed expression studies on various prostate tumor samples using both in situ mRNA hybridization and immunohistochemistry techniques. Our data show a basal level of TSPY expression in normal epithelia, and elevated levels in cancers of increasing Gleason grades. RT-PCR and sequence analyses of TSPY mRNAs from both prostatic and testicular tissues revealed a variety of alternatively spliced variant transcripts whose ORFs would encode either slightly polymorphic isoforms or shortened versions of the TSPY protein. Although the exact nature of these polymorphic TSPY proteins is still unknown, their identification raises the possibility that one or a few of the TSPY isoform(s) may play a role in the development and/or progression of this male-specific cancer.

Table 1. Prostate and testis samples

A. Prostate samples for in situ hybridization and immunohistochemistry

Patient/Sample Number	Age	Gleason Grade[a]	Source
S2312	62	CaP, 3 + 3 = 6	VAMC-SF
S3104	69	CaP, 4 + 5 = 9	VAMC-SF
S2249	64	CaP, 3 + 3 = 6	VAMC-SF
S1748	54	BPH	VAMC-SF
S2011	78	BPH	VAMC-SF
S1050	41	CaP, 5 + 5 = 10	VAMC-SF
S2337	61	CaP, 4 + 5 = 9	VAMC-SF
S482	82	CaP, 5 + 4 = 9	VAMC-SF
S2699	68	CaP, 2 + 3 = 5	VAMC-SF
S1868	58	CaP, 3 + 2 = 5	VAMC-SF

[a] CaP, Prostatic adenocarcinoma.

B. Testis and prostate samples for RT-PCR analysis, cloning and sequencing

Patient/sample number (abbreviated name)	Age	Clinical conditions and/or Gleason grade[a]	Source[b]
16762A1-E (P762CaP)[c]	60	CaP, 3 + 4 = 7	CHTN
16762A2-A (P762NL)[c]	60	BPH of 16762A1-E	CHTN
00-05-A143B (P143B)[c]	80	CaP, 3 + 3 = 6	CHTN
00-07-A218A (P218A)[c]	67	CaP, 3 + 4 = 7	CHTN
00-08-A298B (P298B)	68	CaP, 3 + 4 = 7	CHTN
20475A1A (P475)[c]	64	CaP, 3 + 3 = 6	CHTN
P1 (P1)[c]	72	BPH	CHTN
4001179J (P179)	72	BPH	CHTN
90-02-D020 (T20)	60	normal testis	CHTN
89-09-091 (T91)[c]	65	normal testis	CHTN
89-05-036 (T36)[c]	68	normal testis	CHTN
88-02-045 (T45)[c]	74	testicular mass	CHTN
95-10-H003 (T3)[c]	40	testicular seminoma	CHTN
T1 (T1)[c]	25	normal testis	UCSF
T4 (T4)[c]	36	testicular seminoma (non-tumor portion)	UCSF

[a] CaP, Prostatic adenocarcinoma; BPH, benign prostatic hyperplasia.
[b] CHTN, Co-operative Human Tissue Network; UCSF, Tissue Core, University of California, San Francisco.
[c] cDNA clones were sequenced in this project.

Materials and methods

In situ mRNA hybridization and immunohistochemistry

Five- to seven-micrometer sections were prepared from ten paraformaldehyde-fixed and paraffin-embedded pathological specimens from the archives of the Anatomic Pathology Section, VA Medical Center, San Francisco. The samples were derived from prostatectomy and needle biopsy procedures and were diagnosed to harbor foci of benign prostatic hyperplasia (two cases) and adenocarcinoma (nine cases with Gleason grades ranging from 2+3 to 5+5) by the attending staff pathologists at the VA Medical Center, San Francisco (Table 1A). This and subsequent studies were conducted according to protocols approved by the Institutional Committee on Human Research.

In situ mRNA hybridization was performed with the above prostate tissue sections as previously described (Stelnicki et al., 1998). Briefly, the cDNA of the human TSPY gene was subcloned in the pAR3038 plasmid in either transcription orientation of a T7 promoter. The respective recombinant plasmids were digested with *Bbs*I that truncated the cDNA at the end distal to the T7 promoter. Anti-sense and sense RNAs were synthesized with a riboprobe kit (Roche Biochemicals) using the T7 bacteriophage RNA polymerase in the presence of biotin-16-UTP. The riboprobes were concentrated by ethanol precipitation and were used at 20 μg/ml in the hybridization procedure. The sections were pre-treated with HCl, proteinase K, and acetic anhydride. They were prehybridized and hybridized with the respective biotin-labeled probes in a 50% formamide buffer at 37 °C overnight. The sections were then washed and treated with RNase to eliminate unhybridized single-stranded RNA probes. A tyramide amplification step was used to enhance the hybridization signals (Speel et al., 1999) before the ABC-peroxidase-DAB substrate reactions (Vector Laboratories Inc.). The slides were counter-stained with methyl green and examined under a Zeiss Axiophot photomicroscope.

Immunohistochemistry was performed as previously described using a specific antibody against the human TSPY protein (Lau et al., 2000). The binding of the primary antibody was detected with an affinity-purified goat anti-rabbit IgG and visualized by either ABC-peroxidase or ABC-alkaline phosphatase reagents and substrate kits (Vector Laboratories Inc., Burlingame, CA). For the brown and red signals, the DAB and VECTOR RED kits were used with the ABC-peroxidase and ABC-alkaline phosphatase reagents respectively. An antibody against the human cyclin B1 was a gift from Drs. Catherine Takizawa and David Morgan, UCSF. The monoclonal antibody, PC10, against the proliferating cell nuclear antigen (PCNA) was purchased from Dako Corporation and used according to the procedure provided by the vendor. All three antibodies, TSPY, cyclin B1 and PCNA, had previously been demonstrated to be specific to their respective antigens in immunohistochemistry (Lau et al., 2000). The slides were counter-stained briefly with hematoxylin-eosin and examined with a Zeiss Axiophot photomicroscope as above.

RT-PCR cloning, DNA sequencing and anlaysis

Frozen prostatic and testicular normal and cancer tissues were obtained from either the Cooperative Human Tissue Network or the Tissue Core Laboratory of the Cancer Center at the University of California, San Francisco (Table 1B). Their classifications were based on pathological examination of parallel preparations from the respective samples by attending pathologists at the respective institutions. The prostatic cell line, LNCaP, was obtained from American Type Culture Collection and cultured as before (Lau and Zhang, 2000). Total RNAs were purified by standard procedures using the Trizol Reagent (Invitrogen-Life Technologies), treated with RNase-free RQ1-DNase (Promega Corp.), extracted with phenol-chloroform, precipitated with ethanol, dissolved in DEPC-treated water and stored at −80 °C.

To determine the transcript profiles of TSPY in both prostate and testis samples, cDNAs were synthesized from purified RNA preparations and amplified with specific primers flanking the open reading frame(s) (ORFs) of TSPY gene (Table 2) using a touchdown PCR procedure, as described before (Lau and Zhang, 2000). Amplified cDNA fragments were subcloned in the pGEM-T Easy plasmid using a TA cloning kit (Promega Corp.). 10–15 individual clones containing a TSPY cDNA insert were randomly selected from each sample, purified and sequenced completely from both directions with an ABI 377 sequencer at the DNA Core Laboratory, Howard Hughes Medical Institute, University of California, San Francisco. The cDNA sequences were analyzed and aligned with previously characterized TSPY cDNA and genomic sequences using the MacVector program. They were further aligned with the working draft sequences of the human genome (April, 2003 freeze) using the BLAT program on the Genome Server at the University of California, Santa Cruz. The BLAT program aligns the cDNAs with the respective genomic sequences revealing the structural genes and exon/intron junctions.

Alternate splice junctions were evaluated by RT-PCR amplification of the respective cDNAs using primers derived from the respective flanking exons (Table 2) and the touchdown PCR technique. These primers were composed of end sequences from the two exons at their respective splice junctions. They were used in combination with either a 5′ (HL3) or a 3′ (HL2) TSPY primer in RT-PCR analysis of RNAs derived from prostatic and testicular samples. Only cDNAs derived from transcripts that had undergone the specific RNA processing could be amplified by PCR with the respective primers. The same primer pairs were also used in secondary PCR amplifications of 1:100 diluted products initially obtained by RT-PCR of prostatic and testicular samples using a set of primers (ATG-5 and S-3) flanking the TSPY ORFs. The amplified products were analyzed by agarose gel electrophoresis. Selected fragments were subcloned into plasmid vector and sequenced with an automated sequencer as above.

Results

TSPY is expressed at elevated levels in tumor cells of prostate cancer

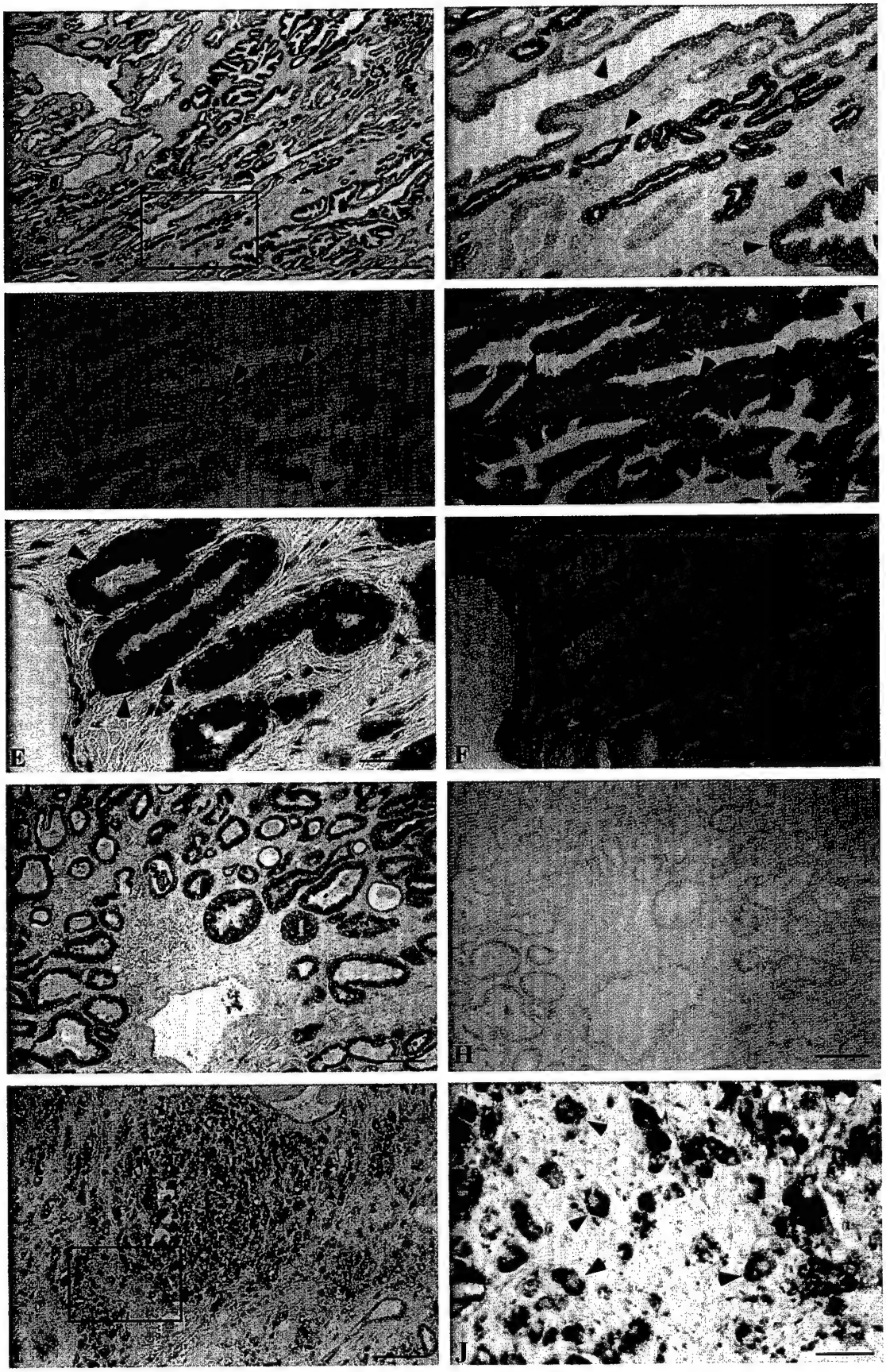
In situ mRNA hybridization was initially used to detect TSPY expression in ten prostate samples containing BPH and/or adenocarcinoma with various Gleason grades (Table 1A). Both antisense and sense biotin-labeled riboprobes were used in the hybridization procedure. We found that incorporating a tyramide amplification step before the substrate development greatly enhanced signal visualization on tissue sections (Speel et al., 1999). Positive signals of varying intensities were observed in all samples with the antisense probe (Fig. 1A, B, C, E, G, I, J) while little or no signals were detected with the sense probe (Fig. 1H). The positive signals were light on normal prostatic structures (Fig. 1B, C, green arrows), moderate on hyperplastic regions (Fig. 1B, orange arrow) and intense on adenocarcinoma (Fig. 1B, C, black arrows). The invading cancerous epithelia (Fig. 1C, black arrows) expressed elevated levels of TSPY mRNA and were clearly discernible among regions of apparently normal morphology (Fig. 1C, D, green arrows). It is interesting to note that such an early invasion of cancerous epithelia was not as readily identifiable on an adjacent section stained only with hematoxylin-eosin (Fig. 1D, black arrows). Under high magnification, the TSPY signals were distributed on the cytoplasm of epithelial cells (Fig. 1E, F, black arrows). TSPY was strongly expressed in adenocarcinoma with increasing grades of malignancy (Fig. 1G, I, J). The signals were also primarily located in the cytoplasm of these tumor cells (e.g. Fig. 1J, black arrows).

To confirm the in situ hybridization results, we extended our study to analyze the TSPY protein expression using a specific polyclonal antibody against a recombinant TSPY protein. Previously, we had used it successfully to detect the TSPY expression in both gonadoblastoma and testicular seminoma sections (Lau, 1999; Lau et al., 2000). Results from this immunohistochemistry study corroborated with those from in situ mRNA hybridization (e.g. Fig. 2A, B and C, D, Fig. 1B, 2J and Fig. 1G, 2E respectively). Again, cancerous cells and/or tumors were clearly visible under low magnification (Fig. 2B). Similar to results from in situ mRNA hybridization, most epithelia

Table 2. Primer sequences used in PCR amplification of cDNAs of TSPY

Primer (abbreviated name)	Sequence
hTSPY-ATG-5 (ATG-5)	5′-ATGCGCCCTGAGGGCTCGCTGA-3′
hTSPY-3 (TSPY-3)	5′-CCATACAATCCACATTTACCCCCTCTTCCTG-3′
hTSPY-S-5 (S-5)	5′-TGGAAGCCCGCGCATGCG-3′
hTSPY-S-3 (S-3)	5′-GACCATGCTGAGTACTGCCGTCCTGCA-3′
PCR4-3 (PCR4-3)	5′-CCTTGAGAATGTTTATTTTTCATTCC-3′
TSPY-Exon1A (1A)	5′-GCACAGGCCTTGGTGGAGCTGGAG-3′
TSPY-Exon1B (1B)	5′-GCACAGGCCTTGCGGGAAAAGATGG-3′
TSPY-Exon1C (1C)	5′-GCACAGGCCTTGATGTCAGCCCTG-3′
Intron4 (Int4)	5′-CGGGAAAGGCCTCATCAGGGCTC-3′
SF-splice (SF)	5′-CATAGGATCTGTCAGGGGACTCAGC-3′
TSPY-HL2 (HL2)	5′-GTCTGCGGCGATAGGCCTCCACTT-3′
TSPY-HL3 (HL3)	5′-TCGGCAGCGGGAAAAGATGGAGCG-3′

Fig. 1. Detection of TSPY expression in prostate samples using in situ mRNA hybridization. (**A–F**) Sample S2249. (**A**) Low magnification view showing a heterogeneous TSPY expression of a prostatic cancer consisting of a mixture of normal (light-staining), hyperplasia (moderate-staining) and tumor (heavy-staining) epithelia. (**B**) Enlargement of the boxed area in (**A**) showing light (green arrow) moderate (orange arrow) and heavy (black arrow) signals. (**C**) Detection of three invading tumor epithelia (black arrows) among the normal prostatic structures (green arrows). (**D**) Hematoxylin-eosin staining of an adjacent section to (**C**). (**E**) High magnification of several tumor epithelia showing cytoplasmic locations of TSPY signals (black arrows). (**F**) Hematoxylin-eosin staining of an adjacent section to (**E**). (**G–H**) Sample S2312. (**G**) In situ hybridization of a tumor using TSPY antisense probe. (**H**) Similar hybridization with a TSPY sense probe on an adjacent section to (**G**). (**I–J**) Sample S3104. (**I**) TSPY mRNA distribution on a highly malignant adenocarcinoma. (**J**) Enlargement of boxed area in (**I**), showing specific expression of TSPY on individual tumor cells. Black arrows indicate examples of cytoplasmic locations of TSPY mRNAs on these cells. The sections were counter-stained with methyl green, except **D** and **F**. The positive signals are brown. Bars represent 800 μm in **A**, 200 μm in **B**, **C**, **D**, **G**, **H** and **I**, and 50 μm in **E**, **F** and **J** respectively.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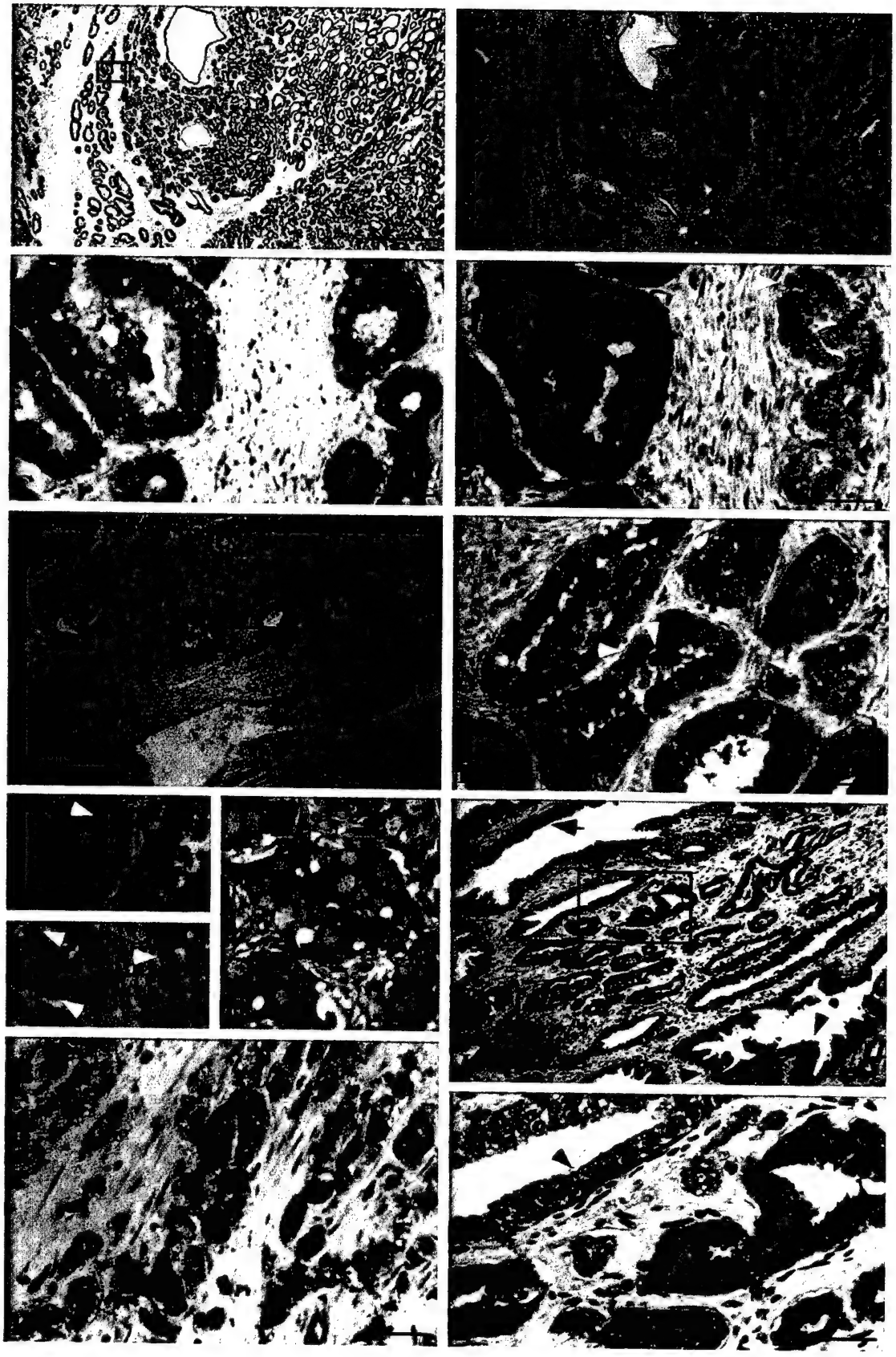

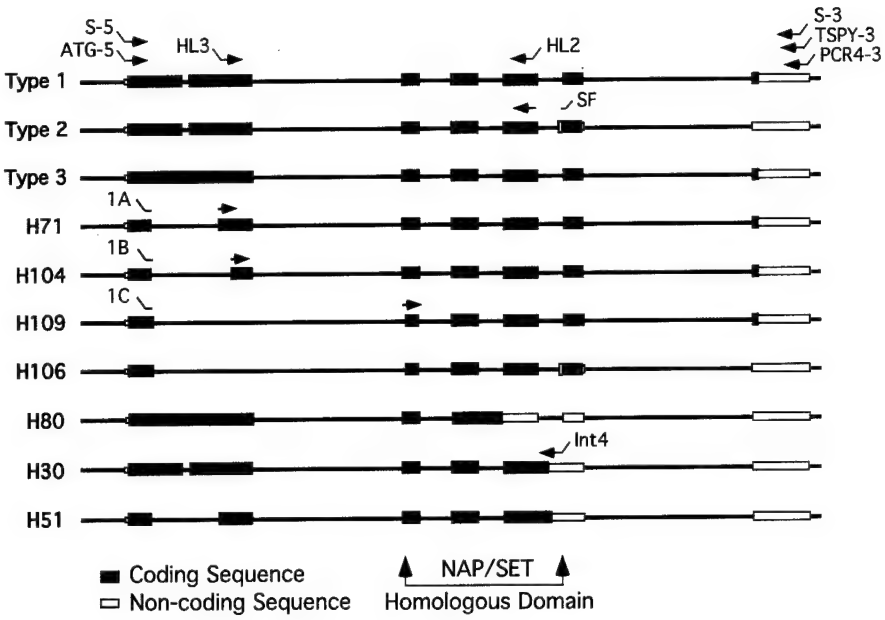

Fig. 3. Diagrammatic illustration of the exon/intron structures and open reading frames of various types of TSPY transcripts. Types 1, 2 and 3 transcripts are previously characterized TSPY transcriptional units. Others are newly identified and characterized in the present study. The positions of the respective primers are as illustrated. The sequences of these primers are listed in Table 2. S-5 and ATG-5 are 5′ primers and S-3, TSPY-3 and PCR4-3 are 3′ primers flanking the ORFs of all known TSPY transcripts. They were used in RT-PCR amplification of cDNAs of the TSPY transcripts from different prostatic and testicular samples. Primers 1A, 1B, 1C, SF and Int4 are specific for the respective splice variants. HL3 and HL2 are 5′ and 3′ primers used in combination with the respective splice junction specific primers in PCR of cDNAs from the tissues. Two vertical arrows indicate the approximate location of the sequence coding for the cyclin B binding domain, similar to those of NAP and SET.

showed varying levels of reactive staining with this TSPY antibody. The signal was low among epithelia of normal morphology (e.g. Fig. 2L, green arrow) and high among those with high-grade cancer (Fig. 2D, E, F, K). Interestingly, some slight differences in immunostaining were observed among adjacent tumor epithelia (Fig. 2D, left and right epithelia) while such differences were not readily detected on in situ mRNA hybridization of a parallel section of the same sample (Fig. 2C). It is uncertain if these minor discrepancies represent true disparity between TSPY mRNA and protein distribution or technical variations between the two techniques used in the studies. Similar to its distribution in gonadoblastoma cells (e.g. Fig. 2I), the TSPY protein was mainly located in the cytoplasm of the prostate cancer cells. However, a significant number of cells showed prominently nuclear staining, especially those of high-grade adenocarcinoma (Fig. 2D, F, G, H, yellow arrows), suggesting that, under certain condition(s), TSPY protein may participate in nuclear activities and/or structural organization of these tumor cells. Immunostaining with specific antibodies against either the proliferating cell nuclear antigen (PCNA) or cyclin B1 showed similar patterns of staining on the same/similar cells/tumors (data not shown). The PCNA was primarily localized on the nuclei while the cyclin B1 signals were detected on both cytoplasm and nuclei of these tumor cells. These observations further suggested that TSPY expression paralleled those of these two proliferating cell markers.

Fig. 2. Corroboration of TSPY expression patterns obtained from in situ hybridization and immunohistochemistry studies. (**A–F**) Sample S2312. (**A**) In situ mRNA hybridization using TSPY antisense probe on a prostate cancer section. (**B**) Immunohistochemistry of an adjacent section using a TSPY specific antibody. (**C**) Enlargement of the boxed area in (**A**). (**D**) Enlargement of the boxed area in (**B**). (**E**) Immunostaining of an adjacent section to Fig. 1G and H. (**F**) Enlargement of boxed area in **E** showing TSPY staining of tumor cells. Although most cells showed cytoplasmic staining, selected cells showed intense staining on their nuclei (yellow arrows), also in **D**, **G** and **H** (Sample S2337). (**I**) Immunostaining of a gonadoblastoma sample (patient 3; Lau et al., 2000) showing prominent cytoplasmic TSPY staining. Blue arrow points to a mitotic cell. (**J**) Heterogeneous expression of TSPY among various epithelia of low/mid grade tumor (Sample S2249), on an adjacent section to that in Fig. 1B. (**K**) Immunostaining of TSPY on a high-grade tumor (Sample S482). (**L**) Enlargement of boxed area in **J**, showing more details of differential staining. Green arrows point to epithelia of apparently normal morphology; orange arrows indicate a potentially hyperplasic epithelium (**J**). Positive signals are brown for in situ hybridization and red for immunohistochemical staining. Bars represent 800 μm in **A** and **B**, 200 μm in **E** and **J**, and 50 μm in **C**, **D**, **F–I**, **K** and **L** respectively.

Differentially processed TSPY transcripts encode a variety of polymorphic proteins

TSPY is a repeated gene with ~35 copies localized on the GBY critical region of the Y chromosome (Skaletsky et al., 2003). Early studies demonstrated that majority of the transcripts are approximately 1.3 kb in size and are derived from 2.8-kb transcriptional units consisting of six exons and five introns (Zhang et al., 1992; Schnieders et al., 1996; Dechend et al., 2000). So far, three major types of transcripts have been identified and characterized from the testis (Fig. 3). The predominant transcript encodes a protein of 308 amino acids (Schnieders et al., 1996) with a predicted molecular weight of 35.1 kDa. We have designated this transcript as type 1 TSPY transcript. A minor transcript is derived from an alternate RNA processing mechanism utilizing an acceptor site located at 11 nucleotides ahead of exon 5 of the predominant transcriptional unit (Zhang et al., 1992). It encodes a protein with 295 amino acids and a calculated molecular weight of 33.3 kDa. It has been designated as type 2 TSPY transcript. A variant of the predominant transcript harbors an in-frame insertion of an 18-nucleotide repeat within the first exon. It adds six amino acids to the ORF resulting in a protein of 314 amino acids with a calculated molecular weight of 35.8 kDa. It has been designated as type 3 TSPY transcript. All three types of TSPY transcripts maintain the same open reading frame, except where the

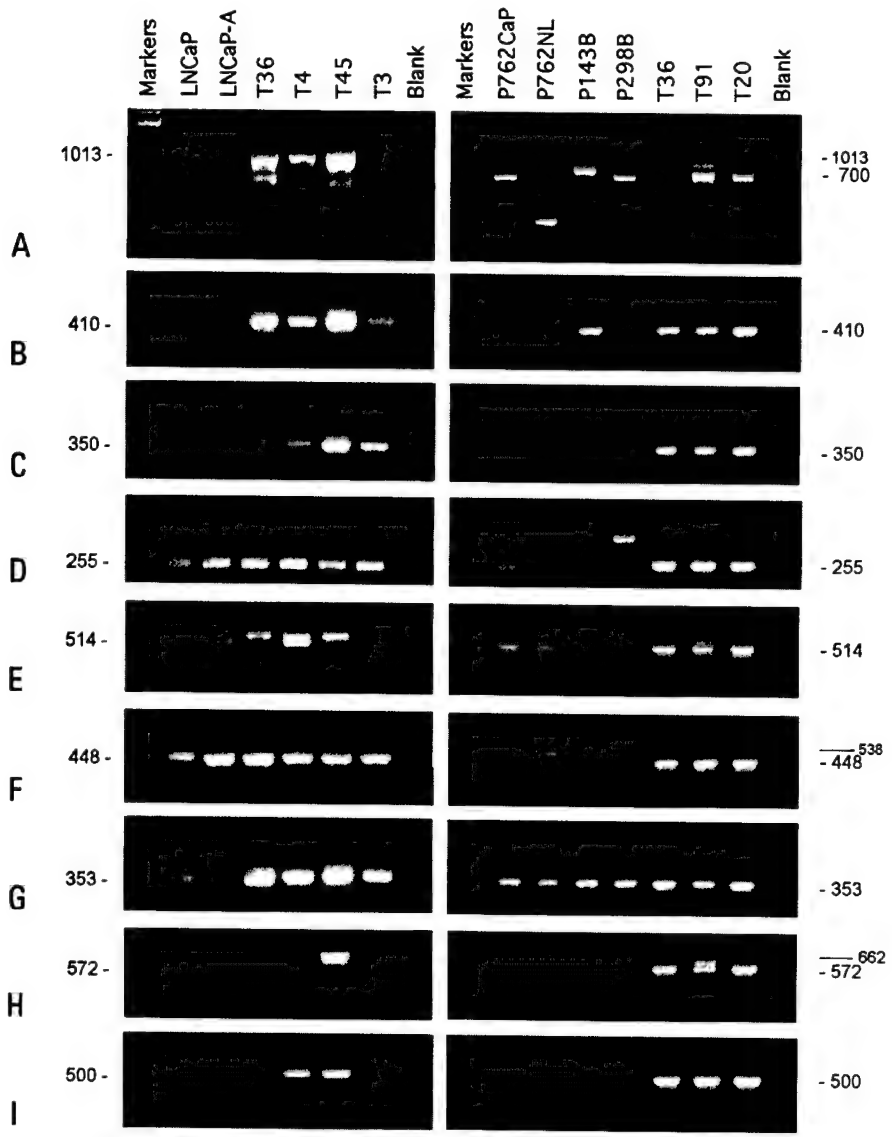

Fig. 4. RT-PCR amplification of TSPY cDNA fragments from prostatic and testicular samples. **(A)** Amplification of cDNA fragments using ATG-5 and S-3 primers flanking the entire ORFs of previously characterized types 1, 2 and 3 transcripts. Primers used in the different RT-PCR amplifications were: **(B)** 1A and HL2; **(C)** 1B and HL2; **(D)** 1C and HL2; **(E)** Int4 and HL3; **(F)** SF and HL3; **(G)** HL3 and HL2; **(H)** 1A and Int4; and **(I)** 1A and SF. See Table 2 for sequences and Fig. 3 for corresponding positions at the transcriptional units. The expected sizes of the respective cDNA fragments are labeled. The numbers in smaller type represent minor fragments amplified with the same primer pairs. See Table 1B for descriptions of the representative prostatic and testicular samples. LNCaP and LNCaP-A were samples from the prostatic cell line, LNCaP. Left panels were derived from direct RT-PCR amplification from respective RNA samples. Right panels were derived from secondary PCR amplification of diluted products obtained initially from RT-PCR of the respective RNA samples using the ATG-5 and S-3 primers. The markers were the 1 kb-plus size markers from Invitrogen-Life Technologies.

changes are present, resulting in slightly polymorphic proteins. They harbor a conserved NAP/SET domain homologous to that shared by some cyclin B binding proteins.

To determine the distribution of the various types of TSPY transcripts in prostatic tissues, a pair of primers (ATG-5 and S-3) flanking the entire ORF of all three types of transcripts were synthesized (Table 2) and used in RT-PCR amplification of the various cDNAs harboring the respective ORFs. Normal and testicular cancer samples were also included in the study. This initial analysis demonstrated a heterogeneous pattern of cDNA products with this primer pair. The predominant bands (e.g. Fig. 4A, left panel) in most testicular samples and the prostatic cell line, LNCaP, seemed to match what were predicted from the previously characterized cDNAs for this gene. Smaller cDNA fragments ranging from 700 to 900 bp were also amplified from these reactions. Significantly these smaller products were especially conspicuous in prostatic samples (e.g. Fig. 4A, right panel). Similar patterns of amplified products were also observed with additional combinations of primer pairs (e.g. Table 2 and Fig. 3, S-5, TSPY-3 and PCR4-3) flanking the entire ORFs. Altering the experimental conditions, such as annealing temperature (up to 68 °C), polymerization time (e.g. 1–3 minutes at 72 °C) and/or cycle number (e.g. 25–45 with five cycle increments) did not significantly repress the amplification of the smaller cDNA fragments (data not shown). These results supported the postulation that they could indeed be derived from a heterogeneous population of TSPY transcripts in the respective tissues.

To determine the sequences of the cDNA fragments, the amplified products from 12 prostatic and testicular samples (Table 1B) and LNCaP cells were subcloned into the plasmid pGEM-T Easy using the TA cloning technique. Over 120 independent clones were purified and sequenced in both directions using an ABI 377 Sequencer and analyzed with the MacVector program in-house and the BLAT program at the Genome Center Server, University of California, Santa Cruz. Table 3 shows

Table 3. BLAT search results for TSPY transcripts[a]

Transcript	Chromosome	Identity (%)	Strand	Start	End
Type 1	Y	99.6	+	9181262	9184056
		99.5	+	9220141	9222934
		99.5	+	9201607	9204402
Type 2	Y	99.6	+	9181262	9184056
		99.5	+	9220141	9222934
		99.5	+	9201607	9204402
Type 3	Y	99.8	+	9260736	9263548
		99.6	+	9140650	9143463
		99.5	+	9240453	9243266
H71	Y	100.0	+	9140696	9143464
		99.9	+	9220187	9222935
		99.9	+	9201653	9204403
H104	Y	99.8	+	9220173	9222935
		99.7	+	9140682	9143463
		99.6	+	9201639	9204403
H109	Y	99.6	+	9240485	9243267
		99.6	+	9181294	9184057
		99.6	+	9140682	9143464
H106	Y	99.5	+	9240485	9243267
		99.5	+	9181294	9184057
		99.5	+	9140682	9143464
H80	Y	100.0	+	9240499	9243267
		100.0	+	9140696	9143464
		99.9	+	9260782	9263549
H30	Y	99.5	+	9181308	9184057
		99.3	+	9220187	9222935
		99.3	+	9201653	9204403
H51	Y	99.9	+	9220187	9222935
		99.9	+	9201653	9204403
		99.9	+	9140696	9143464

[a] April, 2003 freeze of the human genome sequence assembly at Genome Center Server, University California, Santa Cruz; http://genome.ucsc.edu/

the results of these BLAT searches of the human genome sequences. In general, all transcripts seem to have derived from human repeated TSPY transcriptional units at position Yp11.2. In addition to the three types of previously identified TSPY transcripts, there was a complex array of splice variants of TSPY transcripts (Figs. 3 and 5). They can generally be classified into two categories. The first category concerns the first exon in which a cryptic donor site immediately following the codon encoding amino acid residue #29 was used to splice into three different major acceptor sites within exon 1 and 2 of the type 1 TSPY transcript. The first two variants spliced into sequence preceding amino acid residue #117 and #134 respectively in exon 1 (e.g. H71 and H104 respectively, Figs. 3 and 5) while the third variant spliced into sequence preceding amino acid residue #169 in exon 2 (e.g. H109 and H106, Figs. 3 and 5). The cryptic RNA splices resulted in in-frame deletions of 87, 104 and 139 amino acids from their respective ORFs. These transcripts are designated as variant Exon 1A, Exon 1B and Exon 1C respectively. A small cDNA of ~420 bp was apparently the product of alternative splicing events between the exon 1 cryptic donor site and a cryptic acceptor site in the middle of exon 4. Since this transcript contained no apparent protein-coding ORF, it was not studied any further here. The splice variants of exon 1 were primarily type 1 transcripts (e.g. H71, H104 and H109, Figs. 3 and 5) while a few were rare type 2 transcripts (H106, Figs. 3 and 5). Exon 1A seemed to be the

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Type 1    1 MRPEGSLTYRVPERLRQGFCGVGRAAQALVCASAKEGTAFRMEAVQEGAA  50
Type 2    1 MRPEGSLTYRVPERLRQGFCGVGRAAQALVCASAKEGTAFRMEAVQEGAA  50
Type 3    1 MRPEGSLTYRVPERLRQGFCGVGRAAQALVCASAKEGTAFRMEAVQEGAA  50
  H71     1 MRPEGSLTYRVPERLRQGFCGVGRAAQAL----------------------  29
 H104     1 MRPEGSLTYRVPERLRQGFCGVGRAAQAL----------------------  29
 H109     1 MRPEGSLTYRVPERLRQGFCGVGRAAQAL----------------------  29
 H106     1 MRPEGSLTYRVPERLRQGFCGVGRAAQAL----------------------  29
  H80     1 MRPEGSLTYRVPERLRQGFCGVGRAAQALVCASAKEGTAFRMEAVQEGAA  50
  H30     1 MRPEGSLTYRVPERLRQGFCGVGRAAQALVCASAKEGTAFRMEAVQEGAA  50
  H51     1 MRPEGSLTYRVPERLRQGFCGVGRAAQAL----------------------  29

Type 1   51 GVESEQAALGEEAVLLLDDIMAE------VEVVAEEEGLVERREEAQPRQ  94
Type 2   51 GVESEQAALGEEAVLLLDDIMAE------VEVVAEEEGLVERREEAQPRQ  94
Type 3   51 GVESEQAALGEEAVLLLDDIMAEVEVVAEVEVVAEEEGLVERREEAQPRQ 100
  H71    29 ---------------------------------------------------  29
 H104    29 ---------------------------------------------------  29
 H109    29 ---------------------------------------------------  29
 H106    29 ---------------------------------------------------  29
  H80    29 GVESEQAALGEEAVLLLDDIMAEVEVVAEVEVVAEEEGLVERREEAQRAQ 100
  H30    51 GVESEQAALGEEAVLLLDDIMAE------VEVVAEEEGLVERREEAQRAQ  94
  H51    29 ---------------------------------------------------  29

Type 1   95 QAVPGPGPMTPESALEELLAVQVELEPVNAQARKAFSRQREKMERRRKPH 144
Type 2   95 QAVPGPGPMTPESALEELLAVQVELEPVNAQARKAFSRQREKMERRRKPH 144
Type 3  101 QAVPGPGPMTPESALEELLAVQVELEPVNAQARKAFSRQREKMERRRKPH 150
  H71    29 ----------------------VELEPVNAQARKAFSRQREKMERRRKPH  57
 H104    29 ------------------------------------REKMERRRKPH  40
 H109    29 ---------------------------------------------------  29
 H106    29 ---------------------------------------------------  29
  H80   101 QAVPGPGPMTPESALEELLAVQVELEPVNAQARKAFSRQREKMERRRKPQ 150
  H30    95 QAVPGPGPMTPESALEELLAVQVELEPVNAQSKKAFSRQRENMERTRKPH 144
  H51    29 ----------------------VELEPVNAQARKAFSRQREKMERRRKPH  57

Type 1  145 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 194
Type 2  145 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 194
Type 3  151 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 200
  H71    58 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 107
 H104    41 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVGEEKH  90
 H109    29 ------------------------MSALVTDEDEDMLSYMVSLEVEEEKH  55
 H106    29 ------------------------MSALITDEDEDMLSYMVSLEVEEEKH  55
  H80   151 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 200
  H30   145 LDRIGAVIQSVPGFWPNVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 194
  H51    58 LDRRGAVIQSVPGFWANVIANHPQMSALITDEDEDMLSYMVSLEVEEEKH 107

Type 1  195 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 244
Type 2  195 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 244
Type 3  201 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 250
  H71   108 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 157
 H104    91 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 140
 H109    56 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 105
 H106    56 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 105
  H80   201 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITGDRWLPGWVVEGRWWVDHC 250
  H30   195 PVHLYKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 244
  H51   108 PVHLCKIMLFFRSNPYFQNKVITKEYLVNITEYRASHSTPIEWYPDYEVE 157

Type 1  245 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEILCKDLWRNPLQYYKRMKPP 294
Type 2  245 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAESPDRSYVRTCGAIPCNTTRG 294
Type 3  251 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEILCKDLWRNPLQYYKRMKPP 300
  H71   158 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEILCKDLWRNPLQYYKRMKPP 207
 H104   141 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEILCKDLWRNPLQYYKRMKPP 190
 H109   106 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEILCKDLWRNPLQYYKRMKPP 155
 H106   106 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAESPDRSYVRTCGAIPCNTTRG 155
  H80   251 QRDPAPFPQKLLSL                                     265
  H30   245 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEVSPHWETN             282
  H51   158 AYRRRHHNSSLNFFNWFSDHNFAGSNKIAEVSPHWETN             195

Type 1  295 EEGTETSGDSQLLS  308
Type 2  295 N               295
Type 3  301 EEGTETSGDSQLLSN 315
  H71   208 EEGTETSGDSQLLSN 222
 H104   191 EEGTETSGDSQLLSN 205
 H109   156 EEGTETSGDSQLLSN 170
 H106   156 N               156
  H80   265                 265
  H30   282                 282
  H51   195                 195
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Fig. 5. Protein alignment of type 1, 2, 3 and representative TSPY isoforms predicted from the ORFs of alternatively processed transcripts in prostatic and testicular tissues. H71 and H104 represent proteins encoded by type Exon1A and Exon1B transcripts respectively. H109 and H106 are encoded by type Exon1C transcripts with a type 1 and type 2 carboxyl terminus respectively. H80 is encoded by a type 3 transcript with an unspliced intron 3. H30 is encoded by a type 1 or 2 transcript with an unspliced intron 4. H51 is encoded by a complex transcript involving an Exon1A cryptic splicing at its 5′ end and a skipping of intron 4 at its 3′ terminus. See Fig. 3 for the organization of the corresponding exon/intron junctions and ORFs.

predominant species among these exon 1 variants. The cryptic introns were defined at the donor site by a consensus GT dinucleotide from the valine codon (GTG) at residue #30 while all acceptor sites harbored the AG dinucleotide from a preceding glutamine codon (CAG) at the respective junctions. The calculated molecular weights of the proteins ranged from 18.1 kDa (for H106) to 26.0 kDa (for H71). Significantly, the proteins encoded by the type Exon 1A and 1B transcripts were quite basic with estimated pIs around 8.7 while that encoded by the type Exon 1C transcript would have an estimated pI of 6. The isoelectric points for the TSPY proteins encoded by type 1, 2 and 3 transcripts were around 5. Currently, we have not established a biological function for either these variants or the predominant TSPY protein. However, their differences in protein charge suggest that they might exhibit unique properties. The second category of variant transcripts is a minor one, representing less than 5% of the total TSPY cDNA sequences. It involves the skipping of the small introns, 3 and/or 4, in the RNA processing, resulting in altered ORFs beyond the additional sequence from the intron(s) and slightly different proteins at the carboxyl termini (H80 and H30, Figs. 3 and 5). The calculated molecular weights ranged from 30 to 32 kDa in size and estimated isoelectric points of 4.8. Other rare transcripts might harbor both exon 1 variant(s) and intron skipping in the same transcript (e.g. H51, Figs. 3 and 5). Despite the potential difference(s) in properties, all encoded TSPY proteins, however, still harbor either the whole or a major portion of the NAP/SET domain in their respective ORFs (Fig. 3).

Similar BLAT analysis on the type 3 TSPY transcript suggests that it might also be a product of intron skipping event. The type 3 transcript is extremely rare, representing less than 4% of the total TSPY transcripts. Yet, close to 45% of the TSPY transcriptional units in the human genome database (April, 2003 freeze, Genome Center, University of California, Santa Cruz) contain the 18-bp insertion. Currently, we cannot rule out the possibility that transcriptional units with the 18-bp insert are less active than those without. However, close examination of the 18-bp insert, GTG GAG GTG GTG GCG GAG, suggests that it might be a previously unrecognized small intron harboring the consensus splice junctions of GT and AG at its termini. Hence, it is likely spliced from transcripts originating from transcriptional units containing this 18-bp repeat, resulting in low representation of type 3 transcript in the mature TSPY mRNA population. Alternatively, type 3 transcript can be considered as one that skips this 18-bp intron in the RNA splicing process, as those skipping intron 3 and/or 4, observed in the present study.

To confirm the existence of these splice variant TSPY transcripts, specific primers harboring 12-nucleotide sequences from either ends of the respective splice junctions were used in combination with a common 3′ primer (HL2) in PCR analysis of prostatic and testicular cDNAs (Table 2 and Fig. 3). These cDNAs were synthesized from respective RNA preparations by either reverse transcription alone (Fig. 4, left panel) or RT-PCR amplification with primers flanking the TSPY ORFs (Fig. 4, right panel). To detect type 2 variant and intron-skipping transcripts, additional primers crossing the splice variant exons (i.e. SF) and at intron 4 (i.e. Int4) were used in combination with a 5′ primer (HL3) in similar studies (Table 2 and Fig. 3). Results demonstrated that all variant spliced transcripts were indeed present in both prostatic and testicular samples (Fig. 4B, C, D for Exon1A, 1B and 1C or Fig. 4E, F for type 2 and skipped intron4 transcripts respectively). Further, transcripts with complex splicing patterns, e.g. Exon1A splice and skipped intron 4 (Fig. 4H) or Exon1A and type 2 splices (Fig. 4I), were also detected in these samples. One notable exception was Exon1A and type 2 (SF) splices which was undetectable in prostatic samples (Fig. 4I, right panel), suggesting that such complex RNA processing was either absent in prostatic samples or too infrequent for an effective detection under the experimental conditions. Since specific primers for all variants crossed the respective splice junctions and flanked relatively large introns, successful amplification of the predicted RT-PCR products clearly supports their existence in TSPY transcripts of both prostatic and testicular samples.

Based on the sequencing data, the distributions of transcripts with full length or abbreviated ORFs in testicular and prostatic tissues were somewhat different. For the testicular tissues, 52% of the cDNAs harbored the full length ORFs pertaining to those of the predominant type 1 transcript while the minor type 2 and 3 transcripts represented 11 and 2% of the cDNAs respectively. Approximately 19% of TSPY transcripts were type Exon 1A, 1B and 1C variants. The remaining cDNAs were short ~420-bp non-coding fragments and intron-skipping transcripts with altered TSPY ORFs (e.g. H80 and H30, Fig. 3). For the prostatic samples (including the prostatic cell line), the type 1, 2 and 3 transcripts constituted 37, 3 and 3% respectively of the cDNA population while the exon 1 variants were approximately 34%. The remainder was derived from the intron-skipping rare transcripts (e.g. H80, H30 and H51, Fig. 3) and the non-coding 420-bp cDNA, as observed in the testicular samples.

Discussion

Several genetic studies had clearly demonstrated the existence of the GBY locus, a tumor predisposition or oncogenic locus, on this male-specific chromosome (Page, 1987; Salo et al., 1995; Tsuchiya et al., 1995). Deletion mapping has assigned the GBY locus to a small region within deletion interval 3 on the short arm and possibly deletion interval 4 proximal to the centromere on the long arm of the Y chromosome (Salo et al., 1995; Tsuchiya et al., 1995). Recent completion of the human Y chromosome sequence suggests that there are ~35 copies of TSPY gene arranged tandemly in 20.4-kb highly (>99%) homologous repeat units located at the GBY critical region (contigs 2 and 3 in Skaletsky et al., 2003). The TSPY cluster comprises of ~700-kb of Yp sequence and is the largest and most homogeneous protein-coding tandem array, so far identified in the human genome. A single-copy TSPY gene is also located distal to this TSPY cluster at Yp (contig 1) and another one is possibly located at the proximal region on Yq (contig 5). Besides the TSPY cluster, no other protein-coding and functional genes were identified within the GBY critical region. These new findings hence further support the notion that TSPY

is the gene for GBY (Lau, 1999). Significantly, others and we had demonstrated high levels of TSPY expression in the tumor germ cells in gonadoblastoma (Hildenbrand et al., 1999; Lau et al., 2000), thereby further strengthening its candidacy as GBY. Interestingly we also observed a similarly high level of TSPY expression in cancerous germ cells at various stages of testicular seminoma (Lau et al., 2000). In a recent survey on the expression of 31 Y chromosome genes in BPHs, prostate cancer samples and prostatic cell lines, we showed that TSPY expression was heterogeneous among these prostatic specimens and was stimulated by androgen in the LNCaP cells (Lau and Zhang, 2000). The present studies localized the TSPY expression on the epithelial cells of the prostatic specimens and demonstrated a preferential elevation of its expression on the adenocarcinoma cells of the prostate. These studies, taken together, not only support the hypothesis that TSPY is GBY but also strongly implicate it to play a role(s) in other male-specific cancers, including prostate and testicular cancers.

Currently, it is still uncertain, how TSPY exerts its oncogenic activities in gonadoblastoma, seminoma and prostate cancer. TSPY has been postulated to serve a normal function of directing the spermatogonial cells to enter meiosis in the testis (Schnieders et al., 1996; Lau, 1999). TSPY harbors a cyclin B binding domain, similar to those of SET oncogene and nucleosome assembly proteins (NAP) (Tsuchiya et al., 1995; Schnieders et al., 1996; Lau, 1999). Some members of this protein family are involved in either regulating or modulating cell cycle progression (Altman and Kellogg, 1997; Shin et al., 1999; Chai et al., 2001; Canela et al., 2003; Pandey et al., 2003). It is uncertain what effects TSPY might have when it is aberrantly expressed in cells and/or tissues incapable of entering male meiosis. Could a quantitative difference in TSPY expression in such tissues/cells, e.g. prostatic cells or female germ cells, potentiate a cell cycle progression and abnormal cell proliferation? The elevated levels of TSPY expression observed in tumor cells of gonadoblastoma, testis and prostate cancers seem to support such a dysregulation hypothesis.

Previous studies had identified several types of transcripts coding for slightly polymorphic proteins (Zhang et al., 1992; Schnieders et al., 1996; Dechend et al., 2000). Further, base substitutions had also been reported for type 1 TSPY transcripts, involving codon 45, a silent GTG to GTA polymorphism for Val and codon 195, a CCT to CGT polymorphism substituting Pro with Arg (Dechend et al., 2000). These polymorphisms were also observed in our cDNA sequences, including those derived from alternatively spliced transcripts. Further, we had also identified consistent and new amino acid substitutions involving codons 92 and 93 which altered the codons CCC CGA coding for Pro-Arg to codons CGG GCA coding for Arg-Ala at these residues respectively.

The present study demonstrates a more complex pattern of RNA processing, involving cryptic introns and/or alternative donor and acceptor sites of TSPY transcripts. Most alternatively processed TSPY mRNAs maintain ORFs in-frame with that of the predominant type 1 transcript and encode proteins harboring either the whole or part of the conserved cyclin B binding (NAP/SET) domain. The encoded proteins, however, may exhibit significant differences in both the size and properties. Significantly, deletions in type Exon 1A and 1B transcripts result in ORFs encoding relatively more basic proteins than the predominant ones. It will be interesting to determine what effect(s) the deletion(s) might have on the overall function(s) of the TSPY protein. Although we were successful in detecting the expression of the TSPY proteins in prostate cancer using a specific polyclonal antibody, we are uncertain which forms or what expression levels the respective TSPY proteins were in the cancerous cells. Nevertheless, our data raise the possibility that the variant TSPY proteins might indeed possess different properties from those of the predominant form(s). The relative abundance of TSPY exon 1 variant cDNAs from the prostatic transcripts is an interesting observation. In some prostatic and mostly cancerous tissues, the proportion of abbreviated transcripts could be as high as 95% among the cDNA clones examined. Currently, it is uncertain if they play any role in the multi-step process of human oncogenesis. Nevertheless, the present findings on TSPY dysregulation and differential allelic expression have provided a rationale for further investigation on the potential oncogenic or tumor predisposition role of this Y chromosome gene in gonadoblastoma and testicular and prostate cancers.

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